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Immunoregulatory Idiotypes Stimulate T Helper 1 Cytokine Responses in Experimental *Schistosoma mansoni* Infections[1]

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Inbred male CBA/J mice with chronic *Schistosoma mansoni* infections develop two distinct syndromes. Hypersplenomegaly syndrome (HSS) demonstrates pathologic similarities to the hepatosplenic form of chronic human schistosomiasis, and moderate splenomegaly syndrome (MSS) resembles the asymptomatic intestinal form. Immunoaffinity-purified Abs against *S. mansoni* soluble egg Ags (SEA) from infected patients' sera differ idiotypically according to the donor's clinical form of the disease. We now show that immunoaffinity-purified anti-SEA Abs (Id) from MSS or HSS mice parallel idiotypic preparations of the analogous human clinical form by their differential ability to stimulate the proliferation of anti-Id T cells. MSS Id preparations stimulate spleen cells from either MSS or HSS animals. In contrast, HSS Id does not stimulate spleen cells from either group. In an anti-SEA ELISA, MSS and HSS Id preparations contained comparable levels of IgM and IgG1. However, the MSS Id preparation had higher levels of SEA-specific IgG2a and IgG2b than did HSS Id. The Ig isotypes of these Id preparations suggested differences in cytokine expression patterns. Studies of the cytokine profiles of the spleen cells responding to anti-SEA Id preparations demonstrated that while Id preparations from acutely infected mice stimulate IL-4 and IL-10 production, Id preparations from chronic MSS mice stimulate IFN-γ production. HSS Id did not stimulate the production of any of these cytokines. The possibility that distinct immunoregulatory environments may contribute to the development of MSS and HSS correlates with earlier hypotheses that hepatosplenic pathology results at least in part from a lack of development or expression of appropriate regulatory Ids. *The Journal of Immunology*, 1997, 158: 3800–3804.

Chronic *Schistosoma mansoni* infection in humans is a spectral illness, and patients usually present with one of two generalized clinical forms (1–3). Most schistosomiasis patients with chronic disease develop relatively asymptomatic infections. This clinical form is termed intestinal and is associated with only occasional gastrointestinal discomfort despite the continued presence of adult schistosome worms in the mesenteric vasculature. In contrast, <10% of the infected individuals progress to develop the severe hepatosplenic form of the disease, which is characterized by periportal fibrosis, portal hypertension, collateral circulation, esophageal varices, ascites, and hematemesis. The factors that differentiate whether an individual develops the intestinal or hepatosplenic form of chronic schistosomiasis are poorly understood, but many studies have implicated a role for immunoregulatory mechanisms in this process (3, 4). Patients with the intestinal form of chronic schistosomiasis have diminished PBMC proliferative responses to schistosome soluble egg Ag (SEA)[3] compared with patients who have acute schistosomiasis. In contrast, chronically infected patients who are in the process of developing hepatosplenic disease have continued strong PBMC responses to SEA that do not decrease until very late stages of hepatosplenic disease, when they become anergic. Thus, a failure to regulate the acute stage responses to SEA may contribute to the development of hepatosplenic schistosomiasis (2, 3).

One of the mechanisms that may influence whether individuals develop intestinal or hepatosplenic schistosomiasis is their expression of immunoregulatory Ids on given anti-SEA Abs. Patients with regulated (intestinal) schistosomiasis express Ids on their anti-SEA immunoaffinity-purified Abs that stimulate PBMC responses, while patients with unregulated (hepatosplenic) schistosomiasis do not (5). These differences can also be demonstrated serologically by the extent of their reactivity with polyclonal (5) or monoclonal anti-idiotypic Abs (6) and have been shown to play a role in the regulation of granulomatous inflammation in an in vitro granuloma model (7).

Inbred male CBA/J mice with chronic (20-wk) *S. mansoni* infections also develop two distinct syndromes, hypersplenomegaly syndrome (HSS) or moderate splenomegaly syndrome (MSS) (8). Approximately 20% of mice with chronic infections develop HSS, which is characterized by massive splenomegaly, ascites, thymic atrophy, severe anemia, and cachexia. The histopathologic features of the syndrome are splenic congestion, lymph node plasmocytosis, and extensive liver fibrosis, much of which is periportal. The remaining majority (80%) of mice with chronic infections develop only moderate splenomegaly and appear generally healthy. Thus, the pathologic features of HSS and MSS mice parallel many of those exhibited by hepatosplenic or intestinal patients, respectively. Immunologically, mice with HSS express an anti-SEA Ab idiotypic profile distinct from that expressed on anti-SEA Abs from MSS animals, analogous to the serologic differences between human hepatosplenic or intestinal Ids (8). Similarities were also

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[3] Abbreviations used in this paper: SEA, soluble schistosomal egg antigen; HSS, hypersplenomegaly syndrome; MSS, moderate splenomegaly syndrome; 8 wk Id, MSS Id, HSS Id, preparations of affinity-purified anti-SEA antibodies.

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found in the ability of mouse and human Id preparations to stimulate mouse spleen cells (9) or human PBMC (10) from *S. mansoni*-infected mice or humans, respectively. Apparently, mice and humans infected with *S. mansoni* produce some anti-SEA Abs that share the expression of some major cross-reactive Ids.

In this study we investigated the Ig Id and isotype expression on Id preparations from MSS and HSS mice. The data indicate that although both preparations are generated using the same immunoaffinity columns, MSS Id preparations are highly stimulatory for spleen cells, while HSS Id preparations are not. In addition to expressing different Ids, Id preparations from MSS differed from HSS Id isotypically. Because isotype switching is a cytokine-controlled process (11–14), these data led us to investigate differences in cytokine production by spleen cells after exposure to these Id preparations.

Materials and Methods

Mice

Male CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed in the American Association for Accreditation of Laboratory Animal Care-approved animal care facilities of the Centers for Disease Control and Prevention. They were infected by s.c. injection of 45 cercariae of a Puerto Rican strain of *S. mansoni* that had been maintained in *Biomphalaria glabrata* snails.

SEA and Id preparation

The methods used to prepare SEA were described previously (15). Briefly, *S. mansoni* eggs were isolated by differential centrifugation from homogenized liver tissue of CF-1 mice (Charles Rivers Laboratories, Wilmington, MA) 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. Sera from mice at 8 wk of infection or 20 wk of infection (segregated into MSS and HSS by spleen weight and pathologic characteristics) were collected by cardiac puncture and stored at −20°C. For anti-SEA Ab preparations (16), SEA was coupled to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO). Pooled sera from MSS or HSS mice were passed over this SEA-Sepharose column. Multiclonal anti-SEA Abs (Id) were eluted using 0.1 M glycine-HCl (pH 2.8) and collected into 0.025 M borax for neutralization. The eluates were concentrated and dialyzed against saline, and their protein concentrations were determined.

Isotyping

Ab isotype analyses were performed by specific ELISAs to detect anti-SEA activity. SEA (0.25 μg/well in 0.1 M $NaHCO_3$, pH 9.6) was adsorbed onto flat-bottom Immunolon II microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) overnight. Following a blocking step of PBS with 0.3% Tween-20 (Sigma Chemical Co.), plates were incubated with 2.5 μg/ml of Id. The optimal concentration of Id had been determined by performing serial dilutions and plotting absorbance against concentration. A concentration that fell on the linear portion of this curve was chosen for each isotype and used for all further determinations. Anti-SEA Abs bound to the SEA plate were detected using peroxidase-conjugated, isotype-specific, goat anti-mouse IgM or IgG (Boehringer Mannheim, Indianapolis, IN) or anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgG2b (Fisher Scientific, Pittsburgh, PA). This was followed by the addition of TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The subsequent color reaction was read at 450 nm on a microplate reader (Molecular Devices Corp., Menlo Park, CA).

Lymphocyte proliferation assay

The procedure for assaying mouse lymphoid cell proliferation after stimulation by immunoaffinity anti-SEA Abs preparations was described previously (17). Briefly, spleen cells (5×10^5/well) were cultured for 3 days in flat-bottom 96-well microtiter plates (Costar, Cambridge, MA) in 200 μl of RPMI 1640 (Life Technologies, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum (Life Technologies), 3% penicillin-streptomycin (10,000 U/ml and 10,000 μg/ml stock; Life Technologies), and 2 mM L-glutamine (Life Technologies). Cells were pulsed for the final 8 h of culture with 0.5 μCi [^{3}H]TdR (sp. act., 17 Ci/mM; New England Nuclear, Boston, MA) and harvested onto glass fiber filters, and incorporated radioactivity was counted in a Betaplate (Wallac, Turku, Finland) liquid scintillation counter.

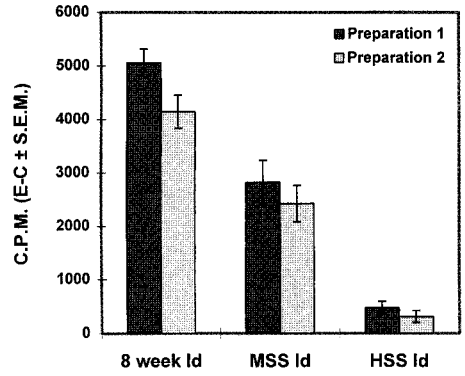

FIGURE 1. Spleen cell responses of 8-wk infected mice ($n = 6$) after exposure to 40 μg/ml of two Id preparations from pooled sera of 8-wk-infected mice (8 wk Id), MSS mice (MSS Id), or HSS mice (HSS Id). The data represent the mean ± SEM countsmin of experimental minus medium control (E−C) counts/min from two experiments using mice from different infection dates. The unstimulated medium control value was 535 ± 37 cpm.

Cytokine production

For analysis of cytokine production, spleen cell suspensions were cultured in triplicate in 96-well U-bottom microtiter plates (Costar) at 2×10^6 cells/well in 0.2 ml/well RPMI 1640 supplemented with 2% fetal bovine serum and 2% penicillin/streptomycin (Life Technologies). Cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2 and stimulated with medium alone, SEA (4 μg/ml), Id (40 μg/ml), or normal mouse IgG (40 μg/ml; Sigma Chemical Co.). Cytokine levels were measured by capture ELISAs in supernatant fluids after 24 h (IL-2 and IL-4) or 48 h (IL-10 and IFN-γ). Culture supernatants were harvested from cells and directly transferred to anti-cytokine ELISA plates that had been coated with 2 (IL-2 and IL-10) or 2.5 (IL-4 and IFNγ) μg/ml anti-mouse cytokine mAb (PharMingen, San Diego, CA) in 0.1 M $NaHCO_3$, pH 9.6. Plates were sealed and incubated with shaking overnight at room temperature. Bound cytokines were detected by sequential incubation with 1 μg/ml biotinylated specific anti-cytokine mAbs (PharMingen), horseradish peroxidase-conjugated streptavidin (Sigma Chemical Co.), and TMB peroxidase substrate solution (Kirkegaard and Perry). Plates were washed three times with PBS and 0.05% Tween-20 (Sigma Chemical Co.) between each step. The change in optical density of each well was measured at 650 nm with a V max kinetic microplate reader (Molecular Devices). Levels of supernatant cytokines were calculated from $\log_2$ dilution curves of homologous recombinant cytokine standards (BioSource International, La Jolla, CA) that were included in each assay.

Results

Anti-SEA preparations from MSS mice stimulate spleen cell proliferation, but those of HSS mice do not. Previous studies in mice have indicated that spleen cell proliferation in response to Id is maximal at 8 wk of infection (18). To compare the stimulatory activity of MSS and HSS Id preparations with that of 8 wk Id preparations, spleen cells from 8 wk infected mice were cultured in the presence of two different Id preparations from 8 wk, MSS, or HSS mice pooled sera. The results (Fig. 1) show that Id preparations from MSS mice stimulate spleen cells from 8-wk infected mice as did 8 wk Id preparations. However, Ids isolated from HSS mouse sera failed to stimulate the proliferation of spleen cells from 8-wk infected mice. The differential stimulatory activities of these Id preparations, which were all generated using the same SEA immunoaffinity column, suggest that the proliferative responses observed are not the result of SEA contamination in these preparations. The order in which these preparations were made using this column was: 8 wk Id preparation 1, MSS Id preparation 1,

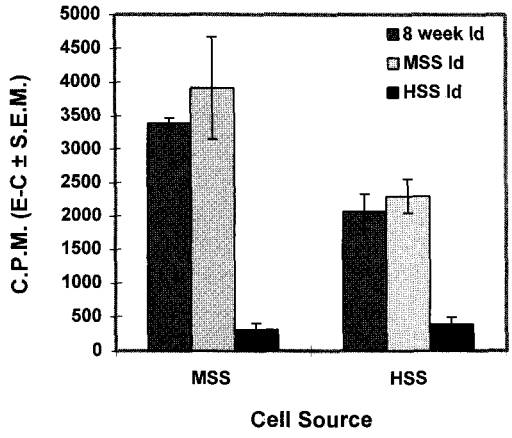

FIGURE 2. Proliferative responses of spleen cells from MSS (n = 12) and HSS (n = 10) mice after exposure to Id preparations from pooled sera of 8-wk-infected mice (8 wk Id), MSS mice (MSS Id), or HSS mice (HSS Id). The data represent the mean ± SEM of experimental minus medium control (E−C) counts/min. The unstimulated medium control value for MSS spleen cells was 1138 ± 340, and that for HSS spleen cells was 1034 ± 637.

HSS Id preparation 1, 8 wk Id preparation 2, MSS Id preparation 2, and HSS Id preparation 2.

We also evaluated the ability of Id preparations to stimulate spleen cells from MSS or HSS mice (Fig. 2). Spleen cells from MSS-infected mice were strongly stimulated by both 8 wk and MSS Id. These same Id preparations stimulated the proliferation of spleen cells from HSS-infected mice. In contrast, HSS Id again, as with cells from mice infected for 8 wk, failed to stimulate spleen cells from either MSS or HSS mice. Spleen cells from uninfected (normal) mice did not respond to any Id preparations (data not shown).

Qualitative differences in isotype profiles of immunoaffinity anti-SEA Abs preparations from MSS and HSS mice

Previously observed differences in idiotypic expression between MSS and HSS mice (8) suggested that there may also be variations in the extent of isotype switching in these animals. To evaluate this, SEA-coated ELISA plates were washed and incubated with anti-SEA Id preparations from MSS or HSS mice. Anti-SEA Id was detected using peroxidase-conjugated IgG fractions of isotype-specific goat anti-mouse IgM or IgG (Fig. 3*a*) or the IgG subclasses IgG1, IgG2a, and IgG2b (Fig. 3*b*). Figure 3*a* demonstrates that MSS and HSS Id preparations contain comparable levels of IgM and IgG. Figure 3*b* shows that levels of IgG1 are also similar in the two groups, but that MSS Id preparations have relatively higher levels of SEA-specific IgG2a and IgG2b than do HSS Id preparations.

MSS Id preparation stimulates IFN-γ production

In mice, isotype switching to IgG2a is at least in part effected by IFN-γ (11–14). The fact that IFN-γ promotes isotype switching to IgG2a led us to investigate differences in cytokine production by spleen cells from acute, MSS, and HSS mice after exposure to Id preparations. Cells from each clinical category of mice were stimulated with the autologous Id preparation, and the culture supernatants obtained were assayed for IL-4, IL-10, and IFN-γ (Fig. 4). While 8-wk Id stimulated IL-4 and IL-10 production by spleen cells from 8-wk infected mice, MSS Id stimulated IFN-γ production by MSS spleen cells. In addition, MSS Id stimulated 8 wk spleen cells to produce IFN-γ (mean ± SEM, 4.82 ± 0.83 U/ml),

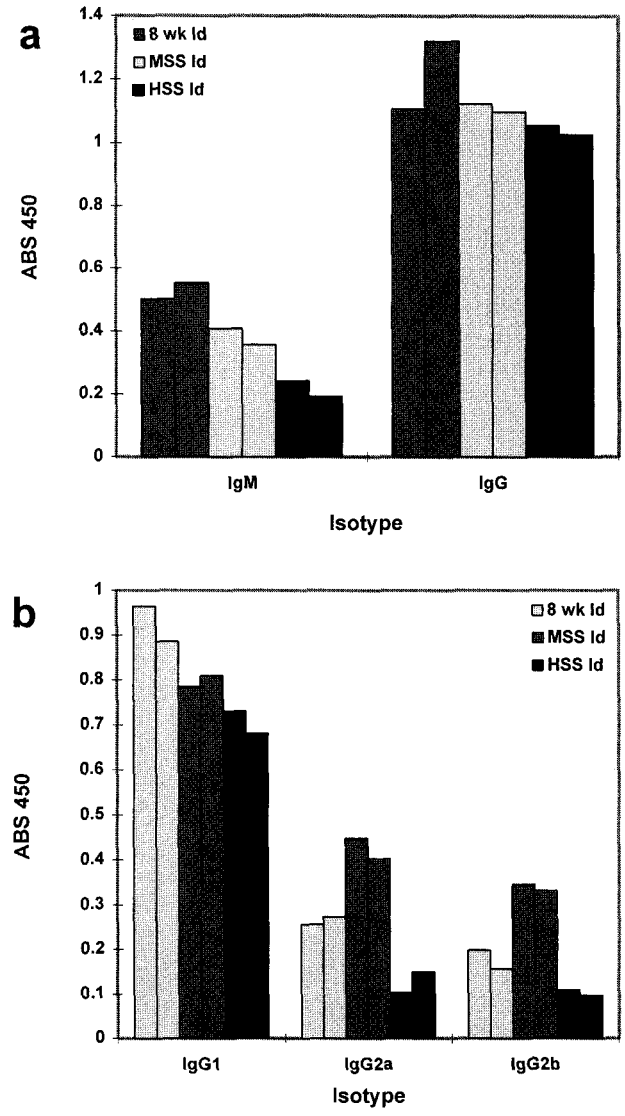

FIGURE 3. SEA-specific isotype profiles of MSS and HSS Id preparations. *a*, IgM and IgG; *b*, IgG subclasses. Data depict absorbances at 450 nm in an SEA-specific ELISA. Double bars represent results from two Id preparations of the same type (see text).

and 8 wk Id stimulated MSS spleen cells to make IL-10 (0.23 ± 0.05 U/ml). No source of spleen cells produced IL-4 or IL-10 or IFN-γ when stimulated by HSS Id or normal mouse Ig (data not shown).

Discussion

In murine and human *S. mansoni* infections, several possible roles of idiotypic/anti-idiotypic interactions have been described, including a protective immune response to schistosomiasis in mice (19–23) and immunopathology of murine (24) and human (25) disease. Mice (10, 17, 18) and humans (5) with *S. mansoni* infections have T lymphocytes that proliferate when exposed to certain preparations of anti-SEA Abs in vitro. Anti-SEA Abs from intestinal and hepatosplenic patients have distinct idiotypic repertoires and anti-idiotypic T lymphocyte stimulatory abilities. These differences in anti-SEA Abs have been interpreted as strong correlative evidence that anti-idiotypic T lymphocytes have a regulatory role in the immunopathogenesis of chronic infections in humans (5, 6).

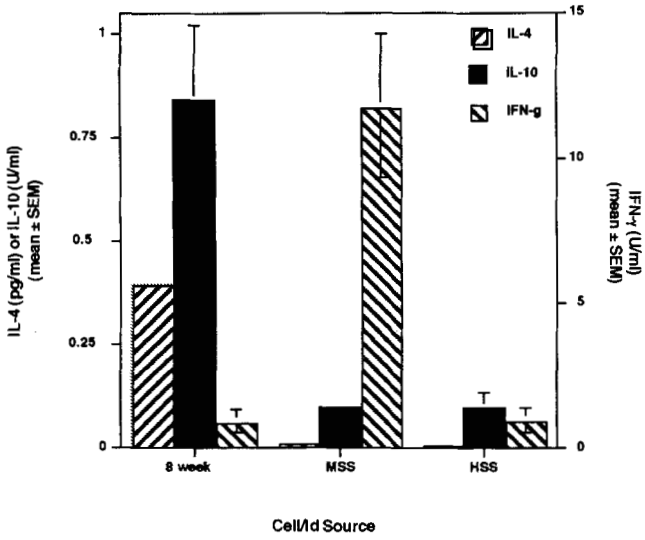

FIGURE 4. IL-4, IL-10, and IFN-γ production by splenic cells from 8-wk infected mice after exposure to 8-wk Id, MSS splenic cells after exposure to MSS Id, and HSS splenic cells after exposure to HSS Id. Data depict the mean ± SEMs of three separate experiments using mice from different infection dates. Each experimental group consisted of cells pooled from spleens of three mice. Analysis of variance (using a two-tailed *t* test) comparing 8-wk and MSS Id/cell combinations indicated $p < 0.0001$ for IL-4, $p = 0.0037$ for IL-10, and $p = 0.0028$ for IFN-γ.

As reported previously (8), male CBA/J mice with chronic *S. mansoni* infections develop two distinct syndromes, MSS and HSS, which differ pathologically and immunologically; these appear to be generally analogous to the intestinal and hepatosplenic clinical forms of chronic schistosomiasis in humans, respectively. The possibility that distinct immunoregulatory environments may contribute to the development of MSS or HSS was supported by the results of competitive ELISA studies, which showed that Ids from HSS and MSS mice are similar to Ids from hepatosplenic and intestinal patients, respectively. Here we present evidence that MSS Ids are stimulatory (as are human intestinal Ids), and HSS Ids are not stimulatory (as human hepatosplenic Ids are not). Thus, in murine as in human schistosome infections, the presence of stimulatory Id correlates with reduced pathology, suggesting that Id stimulation can down-modulate immunopathogenic events in schistosomiasis.

The fact that the HSS Id preparations were isolated on the same SEA immunoaffinity column and did not stimulate spleen cells from 8-wk infected mice indicated that the stimulatory activity of MSS Id as well as that of 8 wk Id preparations is not due to partial SEA contamination or other potential column-related problems. The presence of anti-SEA 8 wk or MSS Id-responsive cells in the spleens of HSS mice could be indicative of an exposure to stimulatory Id earlier in their infections. This might be expected, based on our observations that acute infection in mice can progress to either MSS or HSS.

The proposed differences in activation and regulation between MSS and HSS could also be observed in their anti-SEA isotypes. MSS and HSS Id preparations contain comparable levels of SEA-specific IgM, total IgG, and IgG1, but MSS Id preparations have relatively higher levels of SEA-specific IgG2a and IgG2b than do HSS Id preparations. These same qualitative anti-SEA isotypic differences exist in the sera of MSS and HSS chronic infection mice (26), suggesting that class switching might also be a marker for or play a role in disease regulation.

In the mouse, IgG2a/b production is promoted by IFN-γ, a cytokine associated with Th1 subset activity (11–14). Therefore, the dichotomous pattern of responses in these chronically infected mice imply that MSS mice may express a more Th1-like phenotype than do HSS mice. Immunologic studies of experimental animal models have shown that Th2 responses (IL-4, IL-5, and IL-10) in infected mice become dominant shortly after the onset of egg deposition (27–29). Granulomatous responses in the hepatic tissues shift from vigorous, highly cellular lesions to a compact, less intense response by 20 wk of infection. Several studies of this modulatory stage (8–20 wk) have demonstrated an overall decrease in cytokine levels rather than a strong shift of the types of cytokines produced by SEA or mitogen-stimulated cells as animals have progressed from acute to chronic stages of infection (30–32). Studies of the SEA- or mitogen-stimulated cytokine responses of spleen cells from MSS vs HSS mice have yielded profiles indicating a general decrease in the production of most cytokines, with the only differentiating cytokine features observed to date being HSS mice displaying elevated TNF-α levels in their livers (33) and significantly lower levels of IL-10 being produced by their spleen cells after exposure to SEA.[4] In contrast, we now show strong differences in the cytokine profiles obtained when homologous spleen cells and Ids are used for cytokine production. MSS spleen cells stimulated with MSS Id show a distinct and clear shift to Th1 cytokine production compared with the Th2 cytokine production profile of 8 wk spleen cells stimulated with 8 wk Id. Furthermore, MSS Id stimulated 8 wk spleen cells to produce more IFN-γ than the autologous 8 wk Id, while 8 wk Id caused MSS cells to make more IL-10 than did MSS Id stimulation. These observations bolster the contention that the nature of the Ids obtained from sera of mice with various forms of schistosomiasis express different reactivities, and that these reactivities are responsible for the observed stimulation. Interestingly, IFN-γ has been shown to regulate granulomatous inflammation (32, 34) and is thought to be the effector by which IL-12 down-regulates granuloma and periportal-associated fibrosis (35), which is the observed situation in HSS mice. Along with studies showing that B cell knockout mice (which would be unable to make Id) fail to regulate their granulomas in chronic infection (36), these data reinforce the argument that different stages or forms of murine schistosomiasis produce different anti-SEA idiotypic profiles and that these Ids might play an important role in the modulation of granulomatous hypersensitivity. It appears that at least one mechanism for their initiation or participation in this regulation may be by differential stimulation of IL-4, IL-10, and IFN-γ.

Acknowledgments

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