INTERFERON GAMMA PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS
FROM RESIDENTS OF AN AREA ENDEMIC FOR SCHISTOSOMA MANSONI.

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Abstract

During human schistosomiasis host responses to antigens of various parasite life-cycle stages may contribute to whether the severe, hepatosplenic state develops or the patient remains relatively asymptomatic throughout infection, and may play a role in resistance. This study evaluates in vitro IFN-gamma (IFN\(\gamma\)) production by schistosome antigen-stimulated peripheral blood mononuclear cells (PBMC) from asymptomatic patients, and by PBMCs from apparently uninfected, but Schistosoma mansoni-exposed persons living in areas endemic for S. mansoni. IFN\(\gamma\) production parallels PBMC proliferation in that schistosomal egg antigens stimulate patients' cells poorly, but strongly stimulate PBMCs from persons considered as "endemic normals." This is proportionally true for antigens from adult worms and cercariae. Although asymptomatic patients' cells produced little or no IFN\(\gamma\) in response to the three schistosomal antigenic extracts, their PBMCs produced expected amounts of IFN\(\gamma\) when exposed to PHA. This dichotomy is in contrast to the responses of immunopositive residents living within endemic areas who are not passing eggs, exposed to the risk of schistosome infections, who either are "naturally" resistant, have been infected and "naturally" self-cured, or are carrying adult parasites but are mounting an effective anti-fecundity response. Their PBMCs responded strongly to all three schistosome extracts and PHA, by both lymphocyte proliferation and IFN\(\gamma\) production.
Introduction

Studies of human immune responses to *Schistosoma mansoni* infection have described various mechanisms implicated in the development of resistance to infection, the progression to morbidity, or the regulation of the immune responses involved in either of these host reactivities. Eosinophil-mediated schistosomula killing has been associated with resistance to *S. mansoni* infection in a Kenyan population (BUTTERWORTH, et al. 1975; BUTTERWORTH, et al. 1987), and blocking antibodies correlate inversely with protection (KHALIFE, et al. 1986; DUNNE, et al. 1987). More recently it has been seen that IgE (HAGAN, et al. 1991; RIHET, et al. 1991; DUNNE, et al.) and IgA (GRZYCH, et al. 1993) antibodies correlate directly with resistance, as assessed by reinfection after chemotherapy. Immunoregulatory mechanisms also are thought to contribute to the outcome of disease (COLLEY, et al. 1986; TWEARDY, et al. 1987; COLLEY, 1987). Among these are idiotypic/anti-idiotypic interactions that can influence "in vitro granuloma formation" (COLLEY, et al., 1989; PARRA, et al. 1991). Furthermore, several different schistosomal antigens have been identified as having putative roles in granuloma formation (HARN, et al. 1989; LUKACS and BOROS, 1991), and resistance to infection (BUTTERWORTH, et al., 1987; DESSEIN, et al. 1988; CORREA-OLIVEIRA, et al. 1989; GRZYCH et al. 1993).

Previous studies of cellular immune responses of *S. mansoni*-
infected patients to different schistosomal antigens have demonstrated marked differences between the proliferative responses of patients with different clinical forms of the disease. PBMCs from ambulatory persons with the hepatosplenic or acute forms of schistosomiasis express high responses to soluble egg antigens (SEA), while PBMCs from patients with the asymptomatic/intestinal form show stronger proliferative responses to soluble worm antigen preparation (SWAP) (COLLEY, et al. 1986; BAHIA-OLIVEIRA, et al., 1992). Differences in idiotypic stimulation of PBMCs has also been shown to occur, with anti-SEA-associated idiotypes from intestinal patients being stimulatory to anti-idiotypic T lymphocytes from persons with either intestinal or hepatosplenic disease, but anti-SEA-associated idiotypes isolated from sera of hepatosplenic patients not being stimulatory for cells from patients with any clinical form of the infection (MONTESANO, et al. 1989). Moreover, modulation of in vitro granuloma formation was obtained only when idiotypes from intestinal patients were used to stimulate regulation (DOUGHTY, et al. 1989; PARRA et al. 1991). Thus, the clinical form of schistosomiasis can be directly correlated with immunological findings at the cellular level.

Although the cellular proliferative responses of patients to schistosome antigens have been widely studied, less is known about the cytokines produced by their cells in these cultures. Recent studies have shown that mice chronically infected with S. mansoni display low level IFNγ and IL-2 responses to parasite
antigens and mitogens. This reduced production is related to the duration of infection. Spleen cells from mice with prepatent schistosomiasis produced at least 10 times more IFNγ upon SWAP or SEA stimulation than did animals with chronic infections, implicating patency as the switching point in the predominance of Th1-type to Th2-type responses (MOSMANN and COFFMAN, 1989; GRZYCH, et al.; PEARCE, et al., 1991).

In the current study we present findings of in vitro IFNγ production by PBMCs from infected patients, and "endemic normal" persons (those who live in areas of active transmission of schistosomiasis but have multiple egg-negative stools; (CORREA-OLIVEIRA, et al. 1989) and are putatively immune, upon stimulation with SEA, SWAP, CERC, and PHA. These studies demonstrate that PBMCs from "endemic normal" persons have much higher IFNγ production upon exposure to schistosome antigens than do those of actively infected patients living in the same endemic environment. Since the PBMC IFNγ responses of these 2 groups to PHA are more similar, it appears that patients with patent infections exhibit schistosome antigen-specific defects in their IFNγ production abilities.

Patients, Materials, and Methods

Study Population:

The study population was from the schistosomiasis mansoni endemic area of Siqueira located near metropolitan Belo Horizonte, in the State of Minas Gerais, Brazil. All patients
were diagnosed by repeated fecal examinations using the Kato/Katz method (KATZ, et al. 1972). After passing at least three negative stools, persons were classified as "endemic normals." Upon positive diagnosis of schistosomiasis or other helminthic infections, all patients were treated, independent of their participation in these studies. Persons treated for schistosomiasis received oxamniquine in the standard Brazilian dose of 15 mg/kg for adults and 20 mg/kg for children. The inclusion of all patients in our study groups was preceded by their written consent, or that of their parents or guardians. All residents of the community were also offered medical treatment for other diseases, not necessarily related to the subject being studied, and not contingent upon their participation in this program.

Two groups of participants were studied in this investigation. The first group consisted of pretreatment patients with patent, asymptomatic schistosome infections. We have termed the second study group "endemic normals." These participants live in the schistosomiasis endemic area under study and are known to have periodic contact with water in areas that have infected snails. Their sera contain anti-schistosomal antibodies (CORREA-OLIVEIRA, et al. 1989) and their PBMCs show significant proliferative responses upon stimulation with parasite antigens (see below), but they remain consistently egg-negative upon multiple fecal examinations.

Antigen Preparation:
S. mansoni antigens were prepared according to methods previously described (COLLEY, et al. 1977; GAZZINELLI, et al. 1983). Briefly, adult worms and eggs were collected from outbred Swiss mice infected with the LE strain of S. mansoni maintained in our laboratory. Adult worms, eggs, or cercariae (shed from Biomphalaria glabrata) were suspended in 1.7% NaCl and homogenized on ice with a Potter-Elvejern-type homogenizer equipped with a Teflon pestle until complete disruption, as controlled by microscopic observation. The homogenate was then centrifuged for 1 hr at 50,000g, the supernatant was collected, and its protein content was determined (LOWRY, et al. 1951).

Cellular Proliferation:

Cellular reactivity to S. mansoni antigens was measured using methods previously detailed by us (GAZZINELLI, et al. 1983; COLLEY, et al. 1986). Briefly, PBMCs were separated from heparinized blood by centrifugation on a Ficoll-Hypaque cushion for 40 min at 20 C. They were washed three times with E-MEM (GIBCO, Grand Island, NY), resuspended in RPMI 1640 (GIBCO) at a concentration of 10 x 10^6 cells/ml. For antigen stimulation, 250,000 cells were added per well and for mitogen stimulation, 150,000 cells/well (the final concentrations/ml of culture determined to be optimal were 25 µg of SEA, 25 µg of SWAP, 30 µg of CERC, and 2.5 µl of PHA-P [Difco Laboratories, Detroit, MI]). Triplicate 200 µl cultures contained 5% heat-inactivated normal AB+ human serum. Tritiated thymidine (0.5 µCi/culture; sp.act.
6.7 Ci/mM) was added to cultures on the third d (mitogen stimulation) and sixth d (antigen stimulation) of culture. The cells were harvested 6 h later and proliferation estimated by liquid scintillation spectroscopy. Culture supernatant fluids (80 µl/well) were collected from wells immediately before harvest of the proliferation assay and they were assayed for their IFNγ content.

**IFNγ Assay:**

Supernatant fluids (40 µl) were added to ELISA plates previously coated with 100 µl of a 1:3000 solution of a monoclonal antibody to human IFNγ (Interferon Sciences, New Brunswick, NJ) and blocked with 1% bovine serum albumin (Sigma Chemical Company). The plates were incubated for 1 h at 37C and washed 10 times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween). After the last wash, a polyclonal rabbit anti-human IFNγ antibody was added (1:3000) and the plates were incubated for 1 h at 37C. Following washing with PBS-Tween, a 1:1000 dilution of peroxidase-labeled donkey anti-rabbit immunoglobulin antibody (Jackson Immunoresearch, West Grove, PA) was added to each well and incubated for an additional hr. The plates were again washed and substrate (ABTS, Kirkergaard and Perry laboratories, Gaithersburg, MD) was added (100 µl/well).

For each assay, a standard curve with known concentrations of recombinant human IFNγ (gift of Genentech, San Francisco, CA)
was performed and experimental values were calculated in ng of IFNγ, relative to these values.

Results

PBMC reactivity of patently *S. mansoni*-infected persons to schistosome antigens was assayed by measuring tritiated thymidine incorporation and IFNγ production. Based on proliferative responses, the highest reactivity was observed for SWAP, followed by CERC and then SEA (Fig. 1). The proliferative response of PBMCs of these patients was not different from that previously obtained by our laboratory (COLLEY, et al. 1977; BARSOUM, et al. 1982; GAZZINELLI, et al. 1983; COLLEY, et al. 1986) and others (OTTESEN, et al., 1978; TWEARDY, et al. 1987) where the highest responses observed were usually for SWAP followed by CERC and then SEA. In contrast, PBMC from egg-negative, "endemic normal" individuals demonstrated a higher (p<0.05) mean proliferative response to SEA and to SWAP when compared to those of PBMCs from patently infected patients (Fig. 1). Their responses to CERC and PHA did not differ significantly from those of patients with patent infections (Fig. 1).

The IFNγ data presented in Figure 2 show extremely low levels of production by PBMC from patients with patent infections. These patterns of IFNγ production paralleled the proliferative responses by these cells. The minimal levels of IFNγ measured were not significantly above those obtained from unstimulated cells (data not shown). PHA stimulated both
proliferation (Fig. 1) and IFNγ production (Fig. 2) by the PBMCs of both patients with patent infections and "endemic normals." The inability of the PBMCs from patients who are passing eggs to produce detectable IFNγ was not due to the 6 d time point of cytokine sampling because preliminary studies showed that these cultures also did not contain IFNγ activity at 1 or 3 d after culture (data not shown). Zero-to-minimal levels of IFNγ were obtained when cells of 6 never-infected, Brazilian, norendemic area, control participants were cultured with these schistosomal antigens. Their mean values for exposure to SEA, SWAP, and CERC were: 100, 0, and 133 ng/ml, respectively. In comparison, the mean IFNγ level produced by this group upon PHA-stimulation was 27ng X 10^{-3}/ml. Thus, neither PBMCs from negative controls from nonendemic areas nor those of patients with active *S. mansoni* infections produce high levels of IFNγ upon exposure to schistosomal antigens, but they do produce this cytokine upon mitogen exposure.

In striking contrast to the above finding that patients with patent infections do not produce IFNγ on antigen exposure, Fig. 2 shows that cultures of PBMCs from "endemic normal" individuals incubated with SEA, SWAP, CERC or PHA all yield high levels of this cytokine. This was true whether the culture fluids were collected for examination for IFNγ activity at 1, 3, or 6 d after culture (data not shown).

**Discussion**
Experimental studies based on T cell clones have described two subsets of murine CD4+ T cells that can be characterized, according to cytokine secretion patterns, as Th1 and Th2 (MOSMANN and COFFMAN, 1989), and similar patterns have now been reported for T cell clones from humans (ROMAGNANI 1991; MAGGI, et al. 1992; ROMAGNANI 1992). Sher and colleagues (GRZYCH, et al. 1991; Pearce, et al. 1991) have shown that in schistosome-infected mice, high eosinophil levels and IgE responses (COLLEY, 1975) are under control of the cytokines IL-5 and IL-4, respectively, as produced by Th2-type cells. A role for IgE-mediated hypersensitivity (HAGAN, et al. 1991; RIHET, et al. 1991; DUNNE, et al. 1992), as well as IgA-mediated immunity (GRZYCH, et al. 1993) has been proposed for schistosomiasis. Th1-like responses have been evaluated in experimental schistosomiasis models (FIDEL and BOROS, 1990; PEARCE, et al. 1991; GRZYCH, et al. 1991; HENDERSON, et al. 1991). Mice with pre-patent S. mansoni infections or vaccinated with irradiated cercariae show significant levels of IFNγ and IL-2 production by cultures of stimulated spleen cells, but soon after patency, such mice show dramatic decreases in IFNγ mRNA and IFNγ (PEARCE, et al. 1991; HENDERSON, et al. 1991; HENDERSON, et al. 1992), and precipitous (PEARCE, et al. 1991) or more gradual (FIDEL & BOROS 1990) decreases in IL-2 production. These alterations are followed by concomitant increases in synthesis of IL-4 and IL-5 (PEARCE, et al. 1991).

In agreement with these experimental results, Zwingerberger
and colleagues (1989; 1991) have shown similar low levels of IFNγ and IL-2 production in mitogen and SEA-stimulated PBMC of patients with *S. mansoni*. Their studies showed that mitogen-driven (but not SEA-driven) IFNγ and IL-2 production returned to normal levels within 3 months after drug treatment for asymptomatic infections and within 6 months after therapy for hepatosplenic disease. In parallel with the cytokine measurements, delayed-type hypersensitivity skin tests using SEA were seen to increase after treatment in parallel with the normalization of PBMC IFNγ responses to mitogen (ZWINGERBERGER et al. 1989).

We have extended these studies by assaying IFNγ production by PBMCs of patently-infected patients upon stimulation with SWAP and CERC antigens, and by PBMCs from egg-negative, seropositive persons (CORREA-OLIVEIRA et al. 1989) living in an area where schistosomiasis is endemic ("endemic normals"). We now show that the low levels of IFNγ production by cells from patients with active infections are not restricted to responses to egg antigens but are a general phenomena for antigens from the different developmental stages of the parasite (Fig. 2). However, in contrast to the data obtained by ZWINGERBERGER, et al. (1989; 1991), mitogen-stimulated PBMCs from our egg-positive patients both proliferated and secreted reasonable levels of IFNγ (Figs. 1,2). Possibly differences in patient populations, parasites and/or culture conditions could account for these differences in nonspecific, mitogen-stimulated results.
The data from "endemic normals" provide an interesting contrast when compared with the data from patients with active infections. The former population might be comparable to the "putative-resistant," or "endemic normal" population widely reported on and discussed in filariasis studies (FREEDMAN, et al., 1989; OTTESEN, 1984; PHILIPP, et al. 1988; DAY, 1991), but which is generally ignored in the schistosome literature. It is tempting to speculate about their reported high levels of responsiveness (CORREA-OLIVEIRA, et al. 1989) as it might be related to their apparent state of resistance to reinfection, but such conjecture should follow more complete documentation of their water contact patterns and other immune responses (work in progress). In light of recent studies, an exciting possibility is that "endemic normals" are actually infected, but experiencing efficacious anti-schistosome fecundity and/or embryogenesis immunity (BUSHARA, et al. 1983; Xu, et al. 1993).

Whether the observed suppression in IFNγ production in humans correlates with the modulation of granuloma formation and/or fibrosis, as do decreased IL-4 and IFNγ mRNA patterns in mice (HENDERSON, et al. 1992), remains to be investigated. The cytokine results obtained in experimental models with irradiated cercariae (PEARCE, et al. 1991) and intradermal immunization with S. mansoni paramyosin (PEARCE, et al. 1988) suggest the possibility of a role for IFNγ in the development of resistance to infection. We are now evaluating this question further in longitudinal posttreatment human studies in which reinfected and
nonreinfected persons will be studied and the levels of IFN\(\gamma\) production evaluated.

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*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.*
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Figure Legends

Figure 1. Peripheral blood mononuclear cell proliferation data is given as mean Experimental - Control counts per minute data. Cells were from patients with patent *Schistosoma mansoni* infections (PATENT PATIENTS) (n = 57 for SEA and SWAP; n = 30 for CERC and PHA) or "endemic normal" (ENDEMIC NORMAL) subjects (n = 10 for SEA, SWAP, CERC and PHA). Statistical differences at the p<0.05 level between patient groups exposed to a given antigen are denoted by *.

Figure 2. Production levels of IFNγ produced by peripheral blood mononuclear cells is given as mean ng/ml of culture supernatant fluid. Cells were from patients with patent *Schistosoma mansoni* infections (PATENT PATIENTS) (n = 57 for SEA and SWAP; n = 30 for CERC and PHA), or "endemic normal" (ENDEMIC NORMAL) subjects (n = 10 for SEA, SWAP, CERC and PHA). Statistical differences at the p<0.05 level between patient groups exposed to a given antigen are denoted by *.