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Repeated Exposure Induces Periportal Fibrosis in *Schistosoma mansoni*-Infected Baboons: Role of TGF-β and IL-4[1]

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Recently, we observed that repeated *Schistosoma mansoni* infection and treatment boost Th2-associated cytokines and TGF-β production in baboons. Other studies have shown that some chronically infected baboons develop hepatic fibrosis. Because TGF-β, IL-2, and IL-4 have been shown to participate in development of fibrosis in murine schistosomiasis, the present study examined whether repeated exposure stimulates hepatic fibrosis in olive baboons. To test this hypothesis, animals were exposed to similar numbers of *S. mansoni* cercariae given once or repeatedly. After 19 wk of infection, animals were cured with praziquantel and reinfected once or multiple times. Hepatic granulomatous inflammation and fibrosis were assessed from serial liver biopsies taken at weeks 6, 9, and 16 after reinfection and egg Ag (schistosome egg Ag)-specific cytokine production by PBMC were measured simultaneously. Periportal fibroblast infiltration and extracellular matrix deposition (fibrosis), angiogenesis, and biliary duct hyperplasia developed in some animals. The presence and amount of fibrosis directly correlated with the frequency of exposure. Fibrosis was not associated with adult worm or tissue egg burden. The amount of fibrosis correlated with increased schistosome egg Ag-driven TGF-β at 6, 9, and 16 wk postinfection ($r_s = 0.9$, 0.8, and 0.54, respectively, all $p < 0.01$) and IL-4 production ($p = 0.02$) at 16 wk postinfection and not IFN-γ, IL-2, IL-5, or IL-10. These data suggest that repeated exposure is a risk factor for periportal fibrosis by a mechanism that primes lymphocytes to produce increased levels of profibrotic molecules that include TGF-β and IL-4. *The Journal of Immunology,* 2000, 164: 5337–5343.

Schistosomiasis, a major cause of illness in many tropical or subtropical countries, is a chronic disease that can produce portal hypertension and occasionally death. Adult worms reside in tributaries of the portal vasculature and continuously release ova that pass into the intestine. The portal blood flow, however, carries some eggs to the liver where they are lodged in small vessels of the portal tracts to produce the inflammatory granuloma. It is currently believed that upon death of the ova, the granulomatous response subsides and is followed by tissue repair and fibrosis. In some subjects, fibrosis becomes extended and irreversible and obstructs portal blood flow to produce portal hypertension, esophageal varices, and occasionally death from upper gastrointestinal bleeds. Severe periportal fibrosis, however, develops in <10% of chronically infected individuals (1) for reasons that are not well understood. It has been suggested that periportal fibrosis most often develops in subjects with the heaviest or longest duration of infection (2–5), that have a genetic predisposition (6–8), or have coinfections such as viral hepatitis (9–12).

Studies in murine schistosomiasis demonstrate that the development of fibrosis requires the production of the profibrotic cytokines IL-2, IL-4 (13–15), correlates with TGF-β (TGF-β1) synthesis (16, 17), and is suppressed by IL-12 and IFN-γ (18, 19). Cytokine regulation of hepatic fibrosis and granuloma formation appear to differ (13, 14). Whether these cytokines participate in development of schistosome-induced hepatic fibrosis in humans or nonhuman primates has never been directly tested.

The present study examines the relationship of IL-2, IL-4, TGF-β, and IFN-γ to development of fibrosis in the olive baboon. Baboons are natural hosts for *Schistosoma mansoni* in East Africa (20) and wild-caught baboons with schistosomiasis mansoni have been reported with periportal fibrosis (21). Experimental infections of baboons with *S. mansoni*, however, have not been previously reported to stimulate development of periportal fibrosis (22–24). We previously observed that multiple compared with singly infected animals that are subsequently cured and reinfected produced increased levels of schistosome egg Ag (SEA)[3]-driven TGF-β, IL-4, and IL-2 production by PBMC (25, 26). Based on these observations, we hypothesized that repeatedly infected and treated animals are at an increased risk for development of hepatic fibrosis that correlates with increased IL-2, IL-4, and TGF-β production. To test this hypothesis, olive baboons were repeatedly infected or received an equivalent dose of cercariae and allowed to develop a chronic infection (>19 wk). Animals were subsequently cured with praziquantel and reinfected once or multiple times. Serial liver biopsies were obtained on individual animals following reinfection and were examined for the presence and extent of fibrosis using trichrome staining of fixed tissues. Egg Ag-induced cytokine production by PBMC was examined at 3-wk intervals after reinfection and correlated with the risk of developing fibrosis.

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[3] Abbreviations used in this paper: SEA, schistosome egg Ag; SI, single infection; MI, multiple infection.

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Table I. *Relationship of exposure to the prevalence of hepatic periportal fibrosis in serial liver biopsies of baboons following reinfection after praziquantel treatment*

Group		No. of Animals with Fibrosis (%)		
1° Infection[a]	2° Infection[a]	6 wk PI[b]	9 wk PI	16 wk PI
MI (100 × 10)[c]	MI (100 × 10)	6/6 (100%)[d]	6/6 (100%)	4/6 (67%)
SI (1000 × 1)[c]	SI (1000 × 1)	0/6 (0%)	1/6 (17%)	1/6 (17%)
SI (1000 × 1)	MI (100 × 10)	2/7 (29%)	4/7 (57%)	6/7 (86%)
MI (100 × 10)	SI (1000 × 1)	4/7 (57%)	6/7 (86%)	4/6 (67%)

[a] 1° infection corresponds to initial infection before treatment; 2° infection is reinfection following treatment and cure with praziquantel.
[b] PI, postreinfection.
[c] Animals received 1000 cercariae once or 100 cercariae in each consecutive week for a total of 10 wk.
[d] Animals with >15% of portal tracts with fibrosis are indicated. This cut-off was based on the mean + 2 SD ($n = 5$) from liver biopsies of age- and sex-matched wild-caught baboons without schistosomiasis.

Materials and Methods

Animals, parasites, and infection

A total of 26 juvenile male olive baboons (*Papio cynocephalus anubis*) weighing 6–8 kg were used in this study. The protocols for capture, quarantine, and screening for previous exposure to schistosomiasis have been described in detail before (24). Similarly, the source and life cycle of the parasite (*S. mansoni*) used and the percutaneous infection by the pouch method are as described elsewhere (24). Methods for euthanasia, perfusion of animals for adult worms, and estimates of tissue egg burden have been described previously (24).

Experimental design

The baboons were randomized to four groups of seven animals each (two animals subsequently died during the course of the primary infection and one after treatment and reinfection). The pattern of exposure is shown in Table I. Baboons exposed to a primary infection of either a single dose of 1000 *S. mansoni* cercariae (single infection (SI)) or infected weekly with 100 cercaria for 10 wk (multiple infection (MI)). At 19, 27, and 30 wk after infection, baboons were treated with a single dose of praziquantel (60 mg/kg body weight). Repeated treatment was necessary to accomplish a complete cure as determined by sequential negative stool samples over the course of 1 month. At week 34, animals were reinfected as in the primary infection, either with a single dose of 1000 cercariae or 100 cercariae/week for 10 wk (see Table I).

Pathology assessment

Representative liver biopsies were obtained after laparatomy as described previously (24), at weeks 6, 9, and 16 after reinfection. The liver biopsies were fixed in 10% neutral-buffered Formalin, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin or Masson's trichrome.

Fibrosis assessment

Collagen and other extracellular matrix components were observed in sections stained with Masson's trichrome (see Fig. 1). The number of portal triads with fibrosis were counted and expressed as a percentage of the total number of portal triads per tissue section. Only portal triads with a portal vein diameter of <250 μm were considered since bigger portal triads may have connective tissue as part of their normal histological anatomy and could therefore bias our classification. A minimum of 30 portal triads was counted for each animal. The presence of fibrosis was considered in an animal if >15% of portal tracts had fibrosis. This cut-off value was established based on percentage of portal tracts with any evidence of fibrosis (mean + 2 SD, $n = 5$) observed from liver biopsies in age- and sex-matched wild-caught baboons without parasitological or serological evidence of schistosomiasis.

Cytokine assays

PBMC were cultured for cytokine production at 2×10^6/ml in culture medium (RPMI 1640 (RPMI 1640, 10% FCS, 4 mM L-glutamine, 25 mM HEPES, and 80 μg/ml of gentamicin) in 48-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). Media alone, SEA, prepared as described previously (27), was added to cell cultures at a concentration of 5 μg/ml and incubated at 37°C in a humidified atmosphere with 5% CO_2. Supernatants were harvested after 24 h for the measurement of IL-2 and IL-4 and on day 5 for the measurement of IL-5, IL-10, TGF-β, and IFN-γ production. These time points had been previously determined to be optimal for the specific cytokine determinations.

Cytokine measurements were performed using capture ELISA as previously described for IFN-γ, IL-4, and IL-5 (25). For the cytokine assays for IL-10, IL-2, and TGF-β1, the following assays were performed. ELISA plates (Immulon 4; Dynatech, Sterling, VA) were coated with anti-human IL-10 (mAb AHC8102; BioSource International, Camarillo, CA) at 3 μg/ml in phosphatase buffer at pH 9.6 overnight a 4°C. The detecting Ab was biotinylated anti-human IL-10 (mAb AHC7109; BioSource International) added at 0.8 μg/ml for 1 h at 37°C. Coating Ab for IL-2 was mAb 55.111 (R&D Systems, Minneapolis, MN) used at 4 μg/ml followed by the detecting biotinylated mAb BAF202 (PharMingen, Palo Alto, CA) used at 2.5 μg/ml. TGF-β1 was assayed as follows: the coating Ab was mAb MAB240 (R&D Systems) at 2 μg/ml followed by the detecting biotinylated mAb BAF24 at 0.1 μg/ml (R&D Systems). Before assay for TGF-β, samples were activated by a 10-min incubation with 10 μl of 1 N HCl/50-μl culture supernatants followed by neutralization with 1.2 N NaOH/0.05 HEPES. Steptavidin-alkaline phosphatase (1:2000; Jackson ImmunoResearch, West Grove, PA) was used as a conjugate for all of the cytokine ELISAs while phosphatase tablets (Sigma, St. Louis, MO) were used as substrate. Values were obtained from standard curves using human recombinant cytokines and were expressed in picograms per milliliters. Limits of detection were as follows: 50 pg/ml for IL-2, 40 pg/ml for IL-10, and 25 pg/ml for TGF-β.

Statistical analysis

Cytokine levels are expressed as geometric means and differences between means compared by Student's t test of log-transformed data based on the previous observations that log transformation normalized the data. Correlation coefficients between variables was determined by the Spearman rank correlation, r_s.

Results

Repeated exposure predisposes to periportal fibrosis

Gross assessment of liver pathology was conducted at weeks 6, 9, and 16 after reinfection and hepatic granulomatous inflammation was noted in all of the animals. Microscopically, fibrosis was clearly evident as broadened portal spaces with increased fibroblast infiltration and enhanced connective tissue deposition identified by trichrome stain (blue, Fig. 1, *middle* and *right panels*) were compared with portal tracts without fibrosis (Fig. 1, *left panel*). Many affected portal tracts also showed widespread angiogenesis, biliary duct hyperplasia, and thickening of the portal arteries (Fig. 1, *right panel*). In a few animals, severe congestion or hepatocyte swelling with subsequent obliteration of sinusoids was observed. Although some periovular fibrosis developed in a few animals, most fibrosis occurred in the periportal tracts in the absence of ova. The percentage of portal tracts affected ranged from 0 to 46%.

The presence and amount of fibrosis correlated with the frequency of cercarial exposure (Tables I and II). Table I shows the relationship of exposure and the presence or absence of fibrosis in

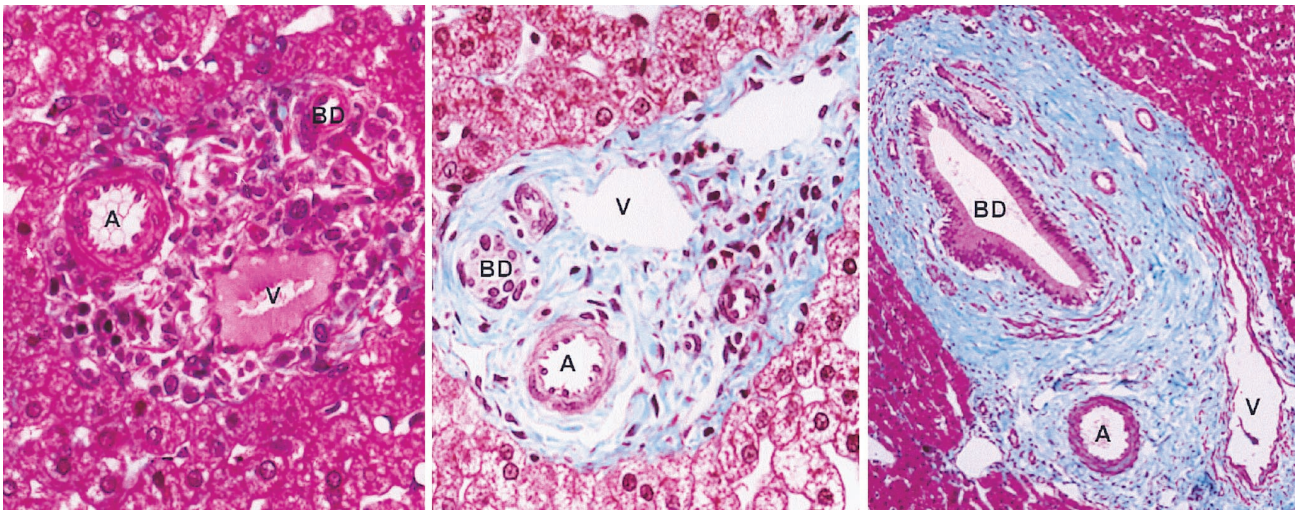

FIGURE 1. Photomicrograph of periportal tracts in an infected animal without fibrosis (*left panel*, from the SI/SI group at ×400 magnification) and with periportal fibrosis (*middle panel*, from the MI/MI group, ×400 magnification). The *right panel* shows the periportal hyperplasia and angiogenesis observed in some animals (×200 magnification). A, artery; V, vein; and BD, bile duct, which constitutes the characteristic structures of portal tracts. Note the significant mononuclear cell infiltrate in the *left panel* without fibrosis.

the different exposure groups. Baboons multiply exposed to cercariae throughout the course of the experiment (MI/MI group) all had fibrosis 6 wk following treatment and reinfection. In contrast, one of six animals that received only two cumulative exposures (SI/SI group) developed fibrosis that was first detected at week 9 after reinfection. The lone affected animal in this group also had the lowest percentage of portal tracts involved, 24% at 9 wk and 17% at 16 wk (Table II). With an increasing number of exposures, the risk of fibrosis increased as illustrated in animals that were multiply infected only after treatment (SI/MI) or before treatment (MI/SI) study groups (Table I). Two animals that received a single infection before treatment followed by repeated infection (SI/MI group) developed fibrosis by 6 wk postinfection (they had a total of six previous exposures by this point) and by 9 wk four animals had fibrosis (after a total of nine exposures). By the time animals had received 11 exposures (a single mass infection before treatment plus 10 weekly exposures after treatment) at week 16, almost all animals had evidence of fibrosis (six of seven animals, Table I). Four of seven animals that were repeatedly exposed only during the primary infection (the MI/SI group) had periportal fibrosis by 6 wk that increased to six of seven animals by week 9.

The amount of fibrosis, as determined by the portion of portal triads affected, also correlated with the frequency of cercarial exposure (Table II). The greatest mean proportion of portal tracts involved was in the MI/MI group at 9 wk postinfection (38%) and lowest in the SI/SI group (0–4%, Table II). Animals with an intermediate cumulative number of exposures during the course of the experiment (the SI/MI and MI/SI groups) also had an intermediate percentage of portal tracts involved. In the two groups with the greatest mean percentage of portal tracts with fibrosis at 9 wk postreinfection (MI/MI and MI/SI groups), the proportion of portal tracts with fibrosis decreased as the infection became chronic by week 16. Indeed, two animals in the MI/MI group and one animal in the MI/SI group that had fibrosis earlier did not show any fibrosis at week 16 after reinfection. Baboon 2069 in the MI/MI group, for example, had the greatest proportion of portal triads affected of all animals throughout the course of the experiment (the high end of range shown in Table II). At 6 wk postreinfection, 42% of its portal tracts had fibrosis that increased to 48% by 9 wk and then diminished to 31%.

Table II. *Mean percentage of portal triads with fibrosis at different times points after infection following praziquantel therapy*

Group	*n*	Mean % Fibrotic Portal Tracts (range)		
		6 wk PI[a]	9 wk PI	16 wk PI
MI/MI	6	32% (20–42%)	38% (28–48%)	22% (0–31%)
SI/SI	6	0%	4% (5–24%)	3% (2–17%)
SI/MI	7	11% (2–21%)	14% (6–28%)	19% (7–29%)
MI/SI	7	15% (4–32%)	23% (7–35%)	18% (8–26%)

[a] PI, postreinfection.

Intensity of infection does not correlate with fibrosis

To examine whether the intensity of infection correlated with the presence of fibrosis in baboons, total worm and tissue egg burdens were measured at the time of sacrifice (16 wk after reinfection) and examined in relationship to the presence of fibrosis at 6, 9, or 16 wk postinfection. An estimate of the intensity of infection before treatment was also determined by weekly measurements of the average number of ova recovered per gram of stool throughout the course of the primary infection. The geometric mean ova per gram of stool for 28 wk before complete cure was equivalent between animals that subsequently developed fibrosis (geometric mean, 20.5; 95% confidence interval, 11.5–30.3) and those that did not develop fibrosis (geometric mean, 20.9; confidence interval, 7.2–33.8). Total worm and tissue egg burden was also similar among animals with and without fibrosis as shown in Table III, assessed at 6 and/or 9 wk postinfection. There was also no relationship between worm and tissue egg burden and the presence or amount of fibrosis determined at 16 wk postreinfection (data not shown). Of note, significantly fewer eggs were recovered from the liver in animals with fibrosis compared with animals without fibrosis. There was also no correlation between amount of fibrosis and adult worm ($r_s = 0.07$, $p = 0.3$) or egg burden ($r_s = -0.21$, $p = 0.12$) among individual animals.

We have previously observed that treatment of singly exposed animals and reinfected animals acquired significantly more adult worms and higher tissue egg burdens compared with multiply exposed animals (25). Animals in the intermediate exposure groups

Table III. *Relationship between hepatic periportal fibrosis and intensity of infection and tissue egg burden*

Fibrosis[a]	*n*	Estimated Total No. of Eggs				
		Adult worms	Large intestine	Small intestine	Liver	Total
Yes	17	222[b]	25,980[b]	957,136	71,835	1,084,983
No	10	299	53,996	1,167,422	113,754*	1,450,903

[a] The presence of fibrosis is based on results obtained at 6 and/or 9 wk postreinfection.
[b] Geometric mean.
*, $p = 0.04$.

have an intermediate number of adult worms and eggs at necropsy (data not shown). Therefore, repeated exposure stimulates partial protection, resulting in a reduced intensity of infection.

The frequency of infection correlates with increased egg Ag-induced TGF-β and IL-4 production by PBMC

Cytokine production by PBMC was examined coincident with liver biopsies. Peak net egg Ag-driven cytokine production was measured at 6 and 9 wk after reinfection (acute infection) and at 13 and 16 wk (termed chronic infection, Fig. 2). Baboons were stratified into three exposure categories: low (2 exposures, SI/SI group, $n = 6$), intermediate (11 exposures, SI/MI and MI/SI groups, $n = 12$), and high (20 exposures, MI/MI group, $n = 6$, Fig. 2). During acute and chronic phases of infection, SEA-induced TGF-β was significantly elevated in the intermediate and high groups compared with the low-exposure groups. During the chronic phase of infection, SEA-induced IL-4 was also significantly elevated in the high group compared with the other exposure groups. There were no significant differences in egg Ag-driven IL-2, IL-5, IL-10, and IFN-γ between the groups (Fig. 2).

Egg-Ag induced cytokine production was also measured in singly ($n = 14$) and multiply ($n = 13$) exposed animals at the same time points after infection described above, but before treatment and reinfection. At 6–9 wk after infection, geometric mean cytokine production (pg/ml ± SEM) in singly vs multiply exposed animals, respectively, was as follows: for IFN-γ, 357 ± 29 vs 253 ± 37; for IL-2, 133 ± 25 vs 71 ± 31; for IL-4, 212 ± 51 vs 98 ± 33; for IL-5, 217 ± 55 vs 101 ± 9, $p = 0.03$; for IL-10, 337 ± 45 vs 115 ± 36, $p = 0.05$; and for TGF-β, 1285 ± 246 vs 976 ± 217. At 13–19 wk postinfection, cytokine levels between the two groups declined by at least 2-fold from the acute phase of the infection and were statistically equivalent between the two exposure groups (data not shown). Egg Ag-induced cytokine responses before treatment did not correlate with subsequent development of fibrosis following reinfection after treatment (data not shown).

Egg Ag-induced TGF-β and IL-4 production by PBMC correlates with the risk of developing periportal fibrosis

Geometric mean net egg Ag-driven TGF-β production by PBMC at 6, 9, and 16 wk significantly correlated with the percentage of portal tracts with fibrosis among individual animals in all exposure groups (Fig. 3, $r_s = 0.9$, $p < 0.001$ at 6 wk, $r_s = 0.8$, $p < 0.001$ at 9 wk, and $r_s = 0.54$, $p = 0.006$ at 16 wk). In contrast, geometric mean net egg Ag-driven IFN-γ production in the same PBMC cultures failed to correlate with fibrosis (Fig. 3). Of note, geometric mean ± SEM egg Ag-driven TGF-β among all animals was 6900 ± 1485 at 6 wk, 7894 ± 1324 at 9 wk, and 5991 ± 838 at 16 wk, which corresponded to a peak in the amount of fibrosis (at 9 wk) and its decline by 16 wk postinfection (Tables I and II and Fig. 3). Spontaneous TGF-β and IFN-γ production did not correlate with the risk of developing fibrosis.

Because SEA-driven IL-4 production was not detected in many animals at 6 and 9 wk postinfection, peak SEA-induced cytokine production was examined at 13 and 16 wk and compared between animals with and without fibrosis. Egg Ag-induced IL-4 production was 12-fold higher among animals with fibrosis after reinfection (geometric mean, 70.4 pg/ml) compared with animals without fibrosis (Fig. 4, geometric mean, 6.2 pg/ml, $p = 0.02$). The other cytokines measured showed no association with the presence or

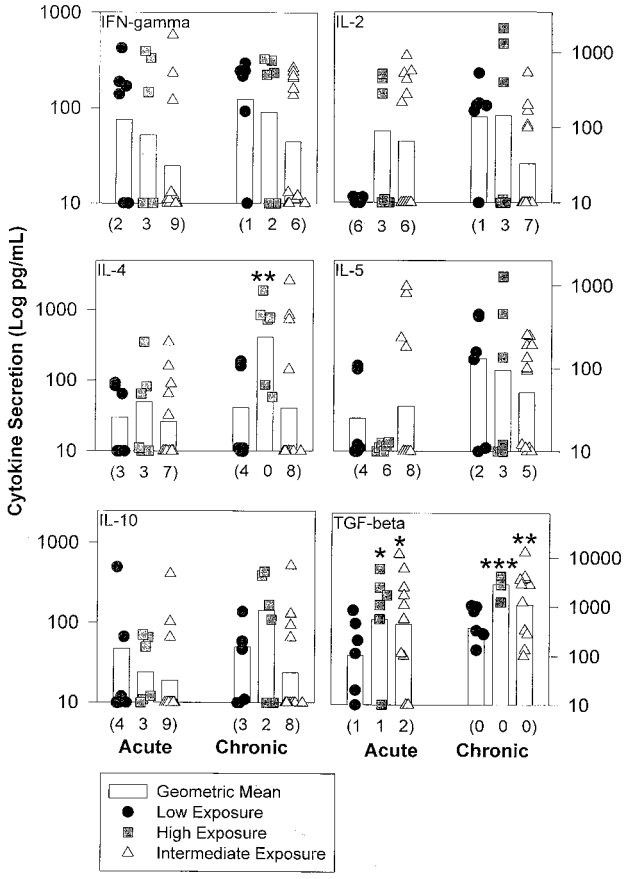

FIGURE 2. The effect of exposure on egg Ag-driven cytokine production following reinfection. Animals were stratified into three exposure categories: low (2 exposures, SI/SI group), intermediate (11 exposures, SI/MI and MI/SI groups), and high (20 exposures, MI/MI group). Acute represents peak cytokine production by PBMC at 6 or 9 wk postinfection and chronic refers to peak cytokine production by PBMC 13 or 16 wk postinfection. Each point represents the mean net cytokine production of duplicate or triplicate cultures of a single animal. Bars represent the geometric mean. Values in parentheses indicate the number of animals that did not have detectable cytokine production. Asterisks represent significant differences between the intermediate- or high-exposure groups compared with the low-exposure group based on Student's *t* test of log-transformed data. *, $p < 0.05$, **, $p < 0.01$

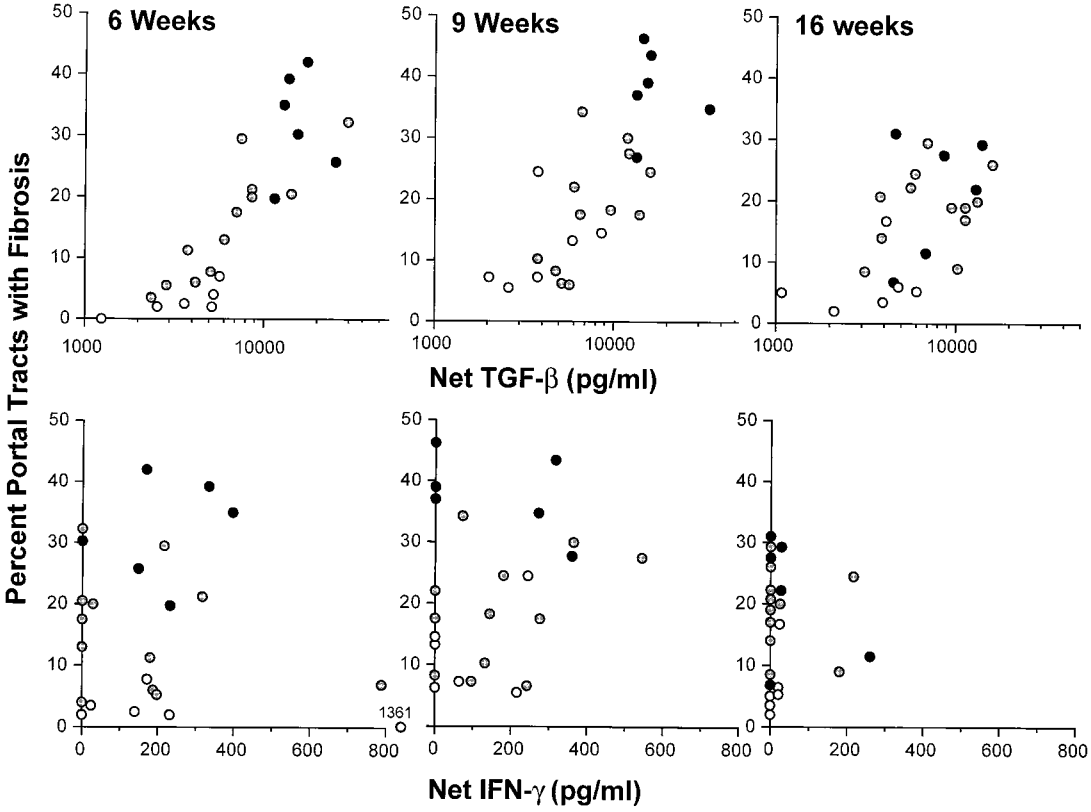

FIGURE 3. The amount of periportal fibrosis correlates with SEA-induced TGF-β production after reinfection. The percentage of portal tracts with fibrosis and corresponding mean net SEA-induced TGF-β production (*upper panels*) and IFN-γ production (*lower panels*) by PBMC (triplicate cultures) are shown for individual baboons at 6, 9, and 16 wk following reinfection after treatment. Open circles represent low exposure, shaded circles represent intermediate exposure, and filled circles represent high-exposure groups. The correlation between percent fibrosis and TGF-β production was significant at 6 ($r_s = 0.9$, $p < 0.001$), 9 ($r_s = 0.8$, $p < 0.001$), and 16 wk ($r_s = 0.54$, $p = 0.006$). There was no significant correlation between the amount of fibrosis and IFN-γ production.

absence of fibrosis. The geometric mean ± SEM egg Ag-induced cytokine production with and without fibrosis at 18 and 16 wk, respectively, was as follows: for IL-2, 33 ± 25 vs 17 ± 21 pg/ml; for IL-5, 122 ± 27 vs 91 ± 43 pg/ml; and for IL-10, 77 ± 45 vs 53 ± 36 pg/ml.

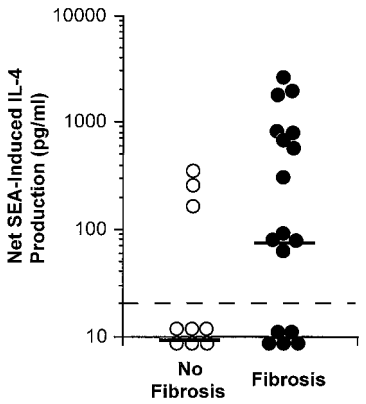

FIGURE 4. Elevated SEA-induced IL-4 production by PBMC after reinfection correlates with development of periportal fibrosis. Animals were considered to have periportal fibrosis if detected at one or more time points after reinfection. Each point represents the geometric mean net peak IL-4 production of triplicates cultures at either 13 or 16 wk postinfection for individual animals. Animals without detectable IL-4 production extended to all sampling time points after infection. Geometric mean IL-4 production is shown as the horizontal bars (no fibrosis, geometric mean = 6.2 and fibrosis, geometric mean = 70.4, $p = 0.02$).

Discussion

The current study shows that repeated cercarial exposure is an important risk factor for development of periportal fibrosis in baboons. The degree of fibrosis correlated with the number of times an animal was exposed and not with the overall number of cercariae, the intensity of infection, or the number of eggs excreted or recovered in tissues. As hypothesized, the development of fibrosis correlated with increased egg Ag-driven TGF-β and IL-4 production, suggesting an etiologic role of these cytokines in the development of fibrosis. These results suggest frequently exposed individuals in endemic populations may be at greater risk of developing fibrosis and measures that reduce frequent infection may help to reduce the overall burden of disease.

Fibrosis was only detectable on histology and there was no associated secondary pathology such as ascites, esophageal varices, or dilation of the portal vein suggestive of increased hepatic portal pressure as seen in advanced periportal fibrosis in humans (3, 28) or some chronically infected chimpanzees (29). None of the baboons studied had more than half of the portal tracts involved with fibrosis. This may have occurred because few hepatic granuloma (usually <5 per cm^3 of liver) were observed compared with mice, even in the most heavily infected animals (>300 worm pairs). The lack of uniform and widespread periportal fibrosis may account for

our failure to detect consistent differences in the amount of fibrosis based on measurement of hydroxyproline (30) between experimental groups (data not shown). We also observed a lack of spatial association with granuloma and the presence of periportal fibrosis. Ova were rarely observed in the portal tracts. Periportal fibrosis with few granuloma has also been observed in humans and animal models of schistosomiasis including chimpanzees, rabbits, and occasionally mice (3, 29, 31). This implies that soluble factors released from the granulomas can induce fibrosis distant from the granuloma itself or parasite-specific lymphocytes preferentially accumulate in the periportal tracts (32) that become activated by Ags released from adults or viable ova elsewhere in the portal vasculature.

The present study also shows that the amount of hepatic fibrosis is greatest around 6–9 wk after reinfection and diminishes by 16 wk. This corresponds to peak granuloma size (6–9 wk) and their decline in size by 16–19 wk, which we have previously reported in baboons (24). This decline in fibrosis was associated with an overall decline in levels of egg Ag-induced cytokine production by PBMC among chronically infected animals (26). These results emphasize that hepatic fibrosis, like granuloma formation, can be actively down-modulated and that this characteristically occurs in most outbred animals.

Detailed studies on the relationship of exposure, intensity of infection and development of pathology for *S. mansoni* infections are very limited in experimental models of schistosomiasis and have been only indirectly studied in humans. Ultrasound examinations of human livers for evidence of high fibrosis grades correlate with the intensity of infection only in children (33–35). Instead, fibrosis in adults has been most strongly associated with gender and duration of infection (5). Studies that have attempted to link exposure (e.g., water contact) to intensity of infection and disease in humans have produced conflicting results (36, 37). Studies in Brazil suggested that exposure is not strongly associated with intensity of infection (36), whereas reports from Africa came to the opposite conclusion (37). Discrepancies in these studies are probably related to difficulties in using water contact as an accurate measure of exposure. Experimental studies of *S. mansoni* infection of nonhuman primates, particularly in the chimpanzee, show that the cumulative number of infective cercariae correlated with the intensity of infection, number of ova in tissues, and development of fibrosis (23, 29). None of these studies, however, examined the relationship between exposure and fibrosis independent from the intensity of infection.

The current studies suggest that egg Ag-induced TGF-β production and, to a lesser extent, IL-4 release contributes to this increased risk of fibrosis. The levels of TGF-β consistently correlated with the risk of developing periportal fibrosis throughout the period of reinfection after treatment. Although SEA-induced IL-4 production observed at 13 and 16 wk postinfection correlated with risk of fibrosis, increased IL-4 production might be expected to be present earlier after reinfection if this cytokine contributed to development of fibrosis. It is possible that we were unable to accurately detect IL-4 in cell culture supernatants because of its rapid consumption or that it develops synergy with other profibrotic molecules, such as TGF-β, at levels that cannot be accurately quantified by the current methods. In contrast, IL-2 and IFN-γ failed to correlate with fibrosis. These cytokines have been implicated in the induction and down-modulation of fibrosis, respectively (13, 18, 19, 38), and the recent observation that severe hepatic fibrosis in humans may be regulated, in part, by a locus that is closely linked to the gene encoding IFN-γR1 (8).

We cannot exclude the possibility that the other cytokines examined also participate in the regulation of fibrosis. IL-2, IL-10, and/or IFN-γ production by PBMC may not reflect cytokine release by lymphocytes within hepatic granuloma or periportal tracts, although we believe this is unlikely for several reasons. First, SEA-induced TGF-β and IL-4 production by PBMC correlated with fibrosis similar to that observed in murine schistosomiasis (14, 16). Second, Ag-specific cytokine production by draining lymph node cells and splenocytes from baboons show the same pattern of cytokine responses as PBMC (our observations). Third, PCR analysis of hepatic tissues containing granulomas tended to show a similar pattern of cytokine responses to that observed in PBMC (our observations). Fourth, a recent report suggested that lymphocytes from the peripheral circulation migrate into and populate granulomas where they undergo cell death by IL deprivation and/or apoptosis (39). Egg Ag-specific lymphocytes also failed to proliferate within the granuloma (39). Therefore, granulomas must contain some egg-specific lymphocytes that arise from other sites that would be represented by PBMC.

Results from the present study suggest that profibrotic mediators and cytokines produced by parasite-specific lymphocytes that are not confined to the granulomas can stimulate fibrosis. Moreover, Ags that stimulate fibrosis may include epitopes shared between different stages of the parasites and possibly Ags unique to larval or adult stages. These possibilities are highlighted by the observation that periportal inflammation and fibrosis occur in the absence of granuloma. The relationship between repeated exposure and development of fibrosis may result from the expansion egg-Ag specific lymphocytes because of shared Ags among developing larvae, adult worms, and ova (40). Some of these shared Ags have been identified as immunogenic carbohydrates that have been shown to preferentially promote T cell differentiation to a Th2-type phenotype (41, 42) and may participate in TGF-β production. Therefore, repeated exposure to developing larvae and those released by adult worms (43) may preferentially expand this population of egg Ag-specific lymphocytes compared with other immunogenic Ags unique to ova that stimulate a more Th1-type phenotype (44). In the case of adult worms, these immunogenic carbohydrates are abundant in the tegument and gut. Killing of adults would markedly enhance Ag release into portal triads to facilitate periportal and perisinusoidal fibrosis observed in the present study. Other studies have shown that mice primed with latex beads coated with only adult worm Ag and/or specific immunogenic carbohydrates like the Lewis X oligosaccharide accelerate hepatic granuloma formation and periportal fibrosis after intraportal inoculation with viable ova (45). Recent studies showing that single sex *S. mansoni* infections of mice can stimulate periportal fibrosis in the absence of ova also indicates that egg Ags may not be necessary to induce fibrosis (46).

Multiple risk factors likely interact to determine whether the host develops periportal fibrosis. This includes duration and intensity of infection modified by host genetics through a common pathway that generates profibrotic cytokines and possibly other molecules. The present study demonstrates that repeated exposure enhances production of profibrotic cytokines that represent an additional risk factor for schistosomiasis-induced hepatic fibrosis. This has important implications for human infections where repeated exposure is common.

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