INFECTION AND IMMUNITY, Nov. 1991, p. 4071–4074
0019-9567/91/114071-04$02.00/0

Vol. 59, No. 11

Analysis of Egg Granuloma Formation in *Schistosoma japonicum*-Infected Mice Treated with Antibodies to Interleukin-5 and Gamma Interferon

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Received 29 April 1991/Accepted 20 August 1991

***Schistosoma japonicum*-infected mice were treated with antibodies to interleukin-5 (IL-5) or gamma interferon (IFN-γ) from week 3 or 4 to week 10 of infection. Neither antibody affected egg production by the parasite, and neither had a consistent effect on the secretion of IFN-γ or IL-5 cell-related cytokines by spleen cells from infected mice. Mice treated with antibody to murine IL-5 had only rare eosinophils in hepatic circumoval granulomas. Granulomas around single eggs were reduced in volume by a third, but hepatic fibrosis was unaffected. Treatment with antibody to murine IFN-γ also reduced the size of granulomas and also did not affect hepatic fibrosis, which was measured as hydroxyproline. Our results, taken together with the studies of others, indicate that a complex interaction of cytokines affects granuloma size and that the size and fibrosis of granulomas are to some extent regulated independently.**

Schistosome worms reside in the portal venous system of the infected host. Most morbidity is caused by the granulomatous reaction to the eggs and the associated hepatic fibrosis, which are largely cell mediated (6, 7). *Schistosoma japonicum* worms begin laying eggs 4 weeks after infection. Circumoval granulomas reach a maximum size 7 weeks after infection, and by 10 weeks newly formed granulomas are immunologically down-regulated and much smaller than at 7 weeks (18). Granulomas are T cell mediated, since small granulomas without eosinophils and with minimal fibrosis are noted at 7 weeks in *S. japonicum*-infected nude mice (7) and normal-size granulomas are present in mice depleted of B cells (6). Nude mice and mice treated with antithymocyte serum develop small granulomas after intravenous injection of *S. japonicum* eggs (17).

Granulomas in murine schistosomiasis japonica develop in concert with augmented Th2- and depressed Th1-cell responses by splenic lymphocytes (22) and thus may not be manifestations of classical delayed hypersensitivity, which is hypothesized to be mediated by Th1 cells (10).

Previous work had suggested an association of eosinophils with hepatic fibrosis in *S. japonicum*-infected nude mice reconstituted with normal spleen cells (8), and Olds and Mahmoud noted a decrease in the size of granulomas around eggs injected intravenously into mice treated with anti-eosinophil serum (17). To further define the role of eosinophils, we have examined the effects of administration of monoclonal neutralizing antibodies to interleukin 5 (IL-5), a cytokine released by Th2 cells and necessary for maturation of eosinophils, and to gamma interferon (IFN-γ), a Th1-cell cytokine, on hepatic granulomas and hepatic fibrosis in mice infected with *S. japonicum*.

MATERIALS AND METHODS

Parasites and snails. *Oncomelania hupensis chui* snails infected with a Philippine (Lowell) strain of *S. japonicum* were received from Yung-san Liang of the Lowell Tropical Medicine Research Institute, Lowell, Mass. Snails were crushed to release cercariae, and C3H/HeN female mice were injected subcutaneously with 15 to 20 cercariae. Mice were received from the Division of Cancer Treatment of the National Cancer Institute, Frederick, Md.

Anticytokine monoclonal antibodies. The TRFK-5 cell line, which produces monoclonal antibody to murine IL-5, and the XMG-2 cell line, which produces antibody to murine IFN-γ, were provided by Robert Coffman of DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, Calif. GL113, which produces an anti-β-galactosidase antibody, was used as a control. Ascites was obtained from pristane-primed nude mice inoculated with 10^7 cells intraperitoneally, and the antibody was partially purified by precipitation with ammonium sulfate.

Evaluation of mice at autopsy. Mice were inoculated intraperitoneally weekly with 1 mg of antibody to IL-5 beginning 3 weeks after infection, 1 week before the worms mature and begin to lay eggs. Injections were continued until 1 week before the mice were killed (7 or 10 weeks after infection). Antibody to IFN-γ was given from the week 4 after infection until 1 week before sacrifice in a dose of 1 or 2 mg weekly or 2 mg twice weekly.

Peripheral eosinophils were counted immediately before sacrifice after dilution of blood in Discombe's solution. After collection of feces over a 24-h period, groups of 5 to 10 mice were killed 7 and 10 weeks after infection by injection of 10 mg of pentobarbital containing 50 U of heparin. Bone marrow was examined as previously described (21). Mice were then perfused to recover adult schistosomes (12). Approximately half of the liver was used for the counting of schistosome eggs following digestion in 4% KOH for 18 h (4). About 200 mg of the liver was used for the estimation of collagen as hydroxyproline by method B of Bergman and Loxley (1), and the remainder was fixed in Bouin-Hollande solution and used to prepare histological sections which were stained with Litt's modification of the Dominici stain (14). A small portion of the small intestine was similarly examined histologically, and the remainder, together with the colon, was digested for the counting of eggs. Feces were

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fixed in 10% neutral buffered formalin, mixed in a Waring blender for 1 min at half speed, caught on nylon cloth (28-μm aperture) to remove fine debris, and then passed through 110-μm-aperture nylon cloth to remove coarse debris. The egg suspension was diluted to about 3 mg of feces per ml, and eggs in duplicate 1-ml volumes in Sedgwick-Rafter chambers were counted.

The diameters of granulomas containing a single egg with a mature miracidium were measured in histologic sections with an ocular micrometer. The diameters of granulomas containing three to five eggs, at least one of which contained a mature embryo, were also measured. An average of 20 single-egg granulomas and 6 multiple-egg granulomas were found in the roughly 2 cm^2 of sectioned liver from each mouse which was examined.

Cytokine responses of treated mice. Spleens from three to five mice were removed aseptically, and cell suspensions were prepared to determine cellular responses to soluble *S. japonicum* egg antigen (20 μg/ml), adult worm antigen (40 μg/ml), and concanavalin A (5 μg/ml) as previously described (22). Briefly, 10^7 cells were cultivated in 2 ml of RPMI medium containing 10% fetal calf serum, and the supernatants were collected after 72 h. The supernatants were frozen, and the amounts of IL-5 and IFN-γ were later determined by enzyme-linked immunosorbent assay (22).

Statistics. Granuloma size, hepatic fibrosis per 10,000 eggs, and the proportion of eggs in the liver decreased with increasing intensity of infection (measured by the number of worm pairs) in these and in previous (5) experiments. These variables were therefore compared by analysis of covariance with worm pairs as the covariate, using logarithms of the variables and of the numbers of worm pairs. Results within treatment groups did not differ significantly in the four experiments done or with the dose of anti-IFN-γ, and the results have been pooled for analysis (see Tables 1 and 2). Mice treated with the control monoclonal antibody, GL113, did not differ significantly from those given saline, and the results for these two groups have also been pooled. Variables which did not change with infection intensity were compared by one-way analysis of variance or by Student's *t* test, results being considered significant for $P \leq 0.05$.

RESULTS

Treatment with antibody to IL-5 or IFN-γ had no evident effect on the number or appearance of the worms, and no difference in the number of eggs per worm pair in the tissues or in the feces was found (Table 1).

Mice treated with anti-IL-5 showed arrested maturation of eosinophils in the bone marrow, peripheral eosinophilia below that of uninfected mice, and almost no eosinophils in hepatic granulomas (Table 2), in which polymorphonuclear neutrophils became the predominant cell type. Eosinophils were also rare in periportal inflammatory infiltrates in these mice. Treatment with anti-IFN-γ had no significant effect on eosinophils in the marrow, blood, or tissues.

Granulomas around single *S. japonicum* eggs in mice treated with anti-IFN-γ or anti-IL-5 had about two-thirds the volume of those in mice treated with GL113, the control monoclonal antibody ($P \leq 0.0001$ at 7 and 10 weeks for anti-IL-5-treated mice and $P \leq 0.05$ for anti-IFN-γ-treated mice at both times by analysis of covariance). The granuloma volume at 10 weeks after infection was about half that at 7 weeks in all groups of mice. Granulomas around clusters of three to five eggs followed a similar trend but did not differ significantly in volume from those in mice treated with GL113. Neither antibody affected hepatic fibrosis (Table 2).

TABLE 1. Parasitologic data

Treatment and time after infection	No. of mice	No. of WP[a]	10^3 Eggs in tissue/WP[a]	No. of fecal eggs/WP[a,b]	% Eggs in liver[a]
7 wk					
GL113 or saline	61	3.5 ± 0.3	48 ± 2	0.7 ± 0.2	28 ± 1
Anti-IL-5	31	3.9 ± 0.4	51 ± 2	1.2 ± 0.1	23 ± 1
Anti-IFN-γ	33	4.6 ± 0.5	47 ± 2	1.2 ± 0.3	24 ± 1
10 wk					
GL113 or saline	41	3.5 ± 0.3	77 ± 3	1.2 ± 0.3	27 ± 1
Anti-IL-5	26	3.7 ± 0.4	82 ± 3	1.4 ± 0.2	24 ± 2
Anti-IFNγ	16	3.0 ± 0.5	77 ± 5	1.5 ± 0.2	30 ± 3

[a] Mean ± standard error of the mean. WP, worm pairs.
[b] Feces were collected from 7 to 13 animals at 7 weeks after infection and from 11 to 18 animals at 10 weeks. All other values were available for nearly all animals.

The continued effectiveness of anti-IL-5 antibody was indicated by the absence of eosinophils in the tissues and peripheral blood. We have no data to indicate the continued effect of anti-IFN-γ antibody treatment, but we were unable to identify antibodies to rat immunoglobulin in anti-IFN-γ-treated mice 7 or 10 weeks after infection and thus have no reason to suspect accelerated clearance of anti-IFN-γ.

Secretion of IL-5 by spleen cells stimulated with concanavalin A was significantly increased at 7 weeks in animals treated with anti-IFN-γ in one experiment but not in a second experiment, and egg antigen-stimulated spleen cells secreted similar amounts of IL-5 in both experiments. IFN-γ secretion was not significantly affected (Table 3).

TABLE 2. Pathogenic findings[a]

Treatment and time after infection	Hepatic fibrosis[b]	Granuloma vol (10^{-3} mm^3)		% Eosinophils in granulomas[c]
		1 egg	3 to 5 eggs	
7 wk				
GL113 or saline	1.29 ± 0.15	31 ± 1.8	87 ± 7.9	39 ± 2
Anti-IL-5	1.07 ± 0.12	20 ± 1.7	80 ± 8.4	0.5 ± 0.1
Anti-IFN-γ	1.16 ± 0.12	22 ± 3.0	67 ± 10.0	38 ± 2
10 wk				
GL113 or saline	2.88 ± 0.18	14 ± 0.9	42 ± 2.6	35 ± 2
Anti-IL-5	2.46 ± 0.19	9 ± 0.8	42 ± 6.2	0.2 ± 0.0
Anti-IFN-γ	2.75 ± 0.22	12 ± 1.2	40 ± 2.9	35 ± 3

[a] All values are expressed as arithmetic mean ± standard error of the mean. See text for statistical analysis.
[b] Normal livers contained a mean of 1.6 μmol of hydroxyproline per liver. Infected mice averaged 6.0 μmol per liver 7 weeks after infection and 16.8 μmol per liver 10 weeks after infection. Data were calculated as (micromoles of hydroxyproline per liver − normal liver hydroxyproline)/10,000 eggs per liver. The number of animals is the same as in Table 1, except that peripheral blood eosinophils and bone marrow were examined in only 7 to 10 infected animals at each time.
[c] Peripheral blood eosinophils (per cubic millimeter, at 7 and 10 weeks, respectively) averaged 453 ± 166 and 835 ± 137 ($P = 0.1$) in mice treated with GL113, 586 ± 169 and 584 ± 276 in those treated with anti-IFN-γ, <20 and 25 ± 12 in anti-IL-5-treated mice, and 85 ± 21 in uninfected mice.

TABLE 3. Cytokine secretion in vitro by spleen cells 7 weeks after infection

Treatment group	IL-5 (ng/ml) after stimulation with[a]:		IFN-γ (ng/ml) after stimulation with:	
	ConA	SEA	ConA	SEA
Expt 1[b]				
GL113	3.06 ± 0.18	2.37 ± 0.05	1.98 ± 0.22	0.63 ± 0.44
Anti-IL-5	3.82 ± 0.09	2.49 ± 0.07	1.39 ± 0.23	0.08 ± 0.00
Anti-IFN-γ	6.36[c] ± 0.31	1.94 ± 0.12	1.46 ± 0.32	0.21 ± 0.14
Uninfected	0.95 ± 0.22	0	5.06 ± 0.49	0.07 ± 0.06
Expt 2[d]				
GL113	2.12	1.44	0.76	0
Anti-IFN-γ	1.79	0.93	0.34	0
Uninfected	0	0	2.97	0.21

[a] ConA, concanavalin A; SEA, *S. japonicum* egg antigen.
[b] Spleens from each of three animals were cultured individually in experiment 1. Values are expressed as mean ± standard error of the mean.
[c] Significantly different from values for the remaining groups by *t* test after Bonferroni's correction for multiple groups.
[d] Means of duplicate measurements for pooled spleen cells were calculated.

DISCUSSION

Our results for mice treated with anti-IL-5 are similar to those for murine *Schistosoma mansoni* infection (21), i.e., there was some reduction of granuloma size but no effect on hepatic fibrosis and no dramatic effect on liver histology, apart from the absence of eosinophils from granulomas and portal infiltrates. It is surprising that the removal of cells as common and as potent as eosinophils had no effect other than to decrease the size of the granulomas. Granulomas around *S. japonicum* eggs in mast cell-deficient W/W^v mice are smaller than those in their congenic partners, an effect which Owhashi et al. ascribed to the eosinophilotactic effect of eosinophil chemotactic factor A in the intact partners (19). Owhashi et al. also reported smaller granulomas in immunoglobulin E-deficient SJA/9 mice than in SJL/J mice (20); however, with mice depleted of B cells (6), we found no change in granuloma size other than decreased modulation of the lesions.

S. japonicum-infected nude mice produce small granulomas without eosinophils and with minimal fibrosis. Injection of cells from normal mice after in vitro depletion of CD4$^+$ or CD4$^+$ and CD8$^+$ cells restored normal-size granulomas without eosinophils or fibrosis, while CD4$^+$ cells partially restored both eosinophils and fibrosis, suggesting that eosinophils might have a role in the fibrotic response (8). The present study shows clearly that tissue eosinophils are not necessary for fibrosis of schistosomal granulomas. The results are similar to those for *S. mansoni*-infected mice treated with anti-IL-5 (21).

Exogenous IFN-γ inhibits collagen synthesis (2) and reduces the size of granulomas around *S. mansoni* antigenic nidi in vitro (13) and around *S. mansoni* eggs in vivo, with a marked decrease in hepatic fibrosis (11). However, in vivo treatment with anti-IFN-γ had no effect on granuloma size or hepatic fibrosis in *S. mansoni*-infected mice (21), and hepatic fibrosis was also not affected in *S. japonicum*-infected mice in the present study. Anti-IFN-γ did significantly decrease granuloma size in *S. japonicum*-infected mice by mechanisms which are not clear. Decreased levels of IFN-γ within granulomas might be expected to favor proliferation of Th2 over that of Th1 cells, suggesting a contribution of Th1 or CD8$^+$ cells to granuloma size. IFN-γ activates macrophages and increases expression and transcription of tumor necrosis factor and class II I-A genes (3), effects which might influence this granulomatous reaction.

Our results, together with those of others, suggest a complex interaction of cytokines with regard to the volume of granulomas around *S. japonicum* eggs. Both immunoglobulin E and eosinophils may play a role, dependent on the Th2-related cytokines IL-4 and IL-5, and the Th1-related cytokine IFN-γ also has an effect. No effect on fibrosis of *S. japonicum* egg granulomas has been shown, although exogenous IFN-γ would be expected to inhibit fibrosis on the basis of the studies noted above.

Most synthesis and accumulation of collagen in the livers of *S. japonicum*-infected mice occur within the granulomas (15), but granuloma size is frequently dissociated from the degree of fibrosis (8, 9, 16, 18). In the present experiments, changes in granuloma size were not accompanied by changes in fibrosis. Anticytokine treatment had no effect on the downward modulation of granuloma size that occurred between 7 and 10 weeks after infection.

ACKNOWLEDGMENTS

We are grateful to Sara Hieny and Patricia Caspar for technical assistance; to Edward Pearce and Jean-Marie Grzych for useful criticisms and shared techniques; to Robert L. Coffman for cell lines TRFK-4, TRFK-5, and XMG-2; to Thomas Kresina for *S. japonicum* egg antigen; and to Sheryl Rathke for editorial assistance.

Snails infected with *S. japonicum* were obtained through NIAID supply contract AI 052590.

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