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Suppressive Effect of Interleukin-4 Neutralization Differs for Granulomas around *Schistosoma mansoni* Eggs Injected into Mice Compared with Those around Eggs Laid in Infected Mice

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The principal pathological manifestation of murine *Schistosoma mansoni* infection is the egg-induced granuloma. Synchronous pulmonary granulomas forming around intravenously injected schistosome eggs are widely used to study the immunopathology of schistosomiasis. A number of anticytokine antibody treatments have a remarkable effect in modulating granulomas in this model but little effect on the size of hepatic granulomas around laid eggs during experimental infection. To examine this discrepancy, we examined the effects of anticytokine antibodies on liver and lung granulomas around injected eggs and around eggs laid during infection in both locations. Anti-interleukin-4 (IL-4) treatment greatly reduced the volume of granulomas around eggs injected into the liver via the portal vein and around eggs injected into the lung via the tail vein. On the contrary, granulomas around eggs laid by worms in either the liver or the lung during the course of infection were not significantly decreased in size by anti-IL-4 treatment. Thus, site is not important for the disparate effects of anti-IL-4 in granuloma formation around injected versus laid eggs. This effect is seen in naive and sensitized animals and is most probably due to differences in the quality of injected eggs versus those laid in situ by the worms.

Egg-induced granulomas that form during *Schistosoma mansoni* infection are CD4-dependent cell-mediated hypersensitivity reactions associated with a strong Th2 response and are the principal pathologic manifestation of the disease in mice (8, 11, 13, 18, 20). The immunoregulatory events involved in granuloma formation are difficult to study in infected mice, as eggs arrive in the liver and gut continuously after the fifth week of infection. Synchronously developing granulomas can be established in the lungs by injecting eggs intravenously (i.v.) (16). By using this model, anti-interleukin-2 (IL-2) and anti-IL-4 have been shown to reduce remarkably the size of granulomas forming around injected eggs or antigen-coated beads (11, 20). On the other hand, these antibodies have only moderate to minimal effects on granulomatous inflammation around eggs laid in infected animals (2, 4, 22). These variations of the effects of anticytokine treatment on granulomas in infected mice and in the lung model may be caused by (i) differences in the microenvironment and/or cytokine production within the various tissues in which eggs are deposited, (ii) greater sensitization of infected mice during granuloma development around laid eggs than during granuloma formation around injected eggs, or (iii) a difference in antigenic properties of injected compared with laid eggs.

In an attempt to resolve some of these issues, we compared granuloma size, mRNA cytokine profiles, and the effect of anticytokine treatment on granuloma formation in the lungs and livers of mice injected with eggs in the tail and portal veins. We also compared these artificial synchronous lesions with those developing around eggs laid during infection of mice with surgically created porto-systemic shunts, which resulted in equal numbers of eggs being deposited in the lungs and liver.

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MATERIALS AND METHODS

Parasites and mice. Cercariae and eggs of the NMRI strain of *S. mansoni* of Puerto Rican origin were from the Biomedical Research Institute (Rockville, Md.). Six-week-old female C3H/HeN mice were purchased from the Division of Cancer Treatment, National Cancer Institute (Frederick, Md.). Generally, each experimental group contained five mice. All experiments except for cytokine assessment in egg-injected livers were done at least twice.

Lung and liver granulomas around laid eggs in infected mice. Mice were exposed to 40 cercariae percutaneously. Four weeks later, the portal vein was partially ligated as previously described (3). In brief, animals were anesthetized with pentobarbital, a midline abdominal incision was made, and the portal vein was partially occluded with a 23-gauge needle (diameter, 0.635 mm) as a stent that was removed after ligation. By this procedure, a porto-systemic shunt is created, and roughly equal numbers of laid eggs will pass to the liver and to the lung. Eight weeks after infection, the mice were sacrificed, and the liver and the lung were processed for histological evaluation and determination of cytokine steady-state mRNA levels.

Lung and liver granulomas around i.v. injected eggs. (i) Isolation of eggs. Eggs were isolated from the livers of mice exposed to 250 cercariae 7 weeks previously. Following perfusion of the liver with citrate saline to recover adult worms, livers were incubated overnight at 4°C and for 1 h at 37°C. They were then blended in a food processor in 1.8% saline, 50 ml per liver. After the coarse debris had been removed through nylon cloth, eggs were isolated by repeated low-speed centrifugation.

(ii) Egg injection. To induce synchronous granulomas in both the lung and the liver, 3,000 eggs were injected in the tail vein and then 3,000 eggs were injected in the portal vein, as previously described (5). Ten and 14 days after injection, animals were sacrificed, and the liver and lung were processed for histological evaluation and cytokine mRNA determination. In some experiments, animals were sensitized by injection of 5,000 eggs intraperitoneally 4 weeks prior to i.v. challenge.

Anti-IL-4 treatment of mice. A neutralizing monoclonal antibody (MAb) against murine IL-4 (11B11) was purchased from Verax Corp. (Lebanon, N.H.). An immunoglobulin G1 (IgG1) control MAb (GL113) directed against *Escherichia coli* β-galactosidase (a gift from John Abrams, DNAX, Palo Alto, Calif.)

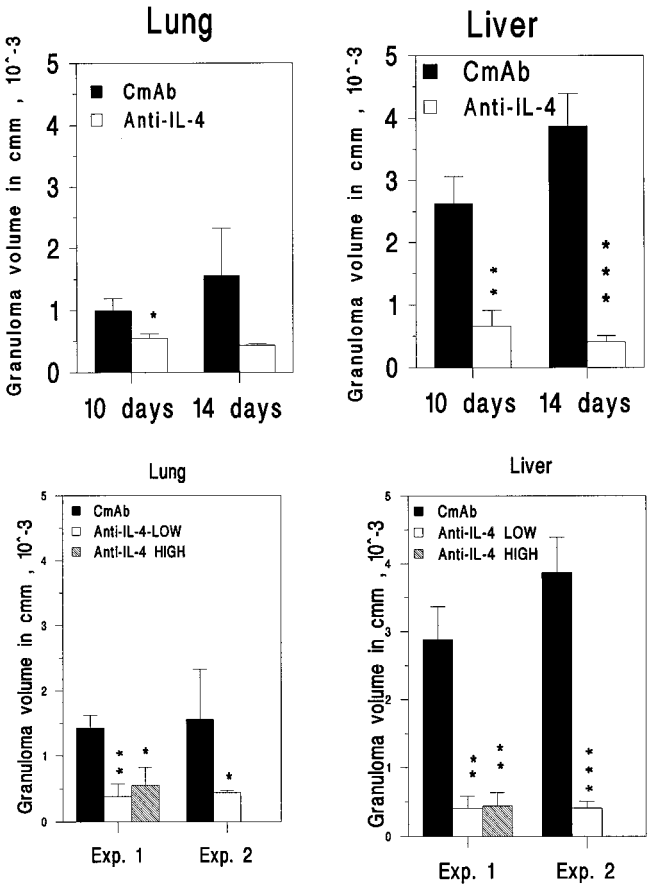

FIG. 1. Anti-IL-4 reduces the granuloma size around injected eggs. Three thousand eggs were injected in the lung or the liver, and the size of the granuloma was determined. (Top) Granuloma volume at days 10 and 14 after egg injection; (bottom) granuloma volume after high and low doses of anti-IL-4. cmm, cubic millimiters; vertical bars, 1 standard error of the mean; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with results in mice treated with control MAb [CmAb]).

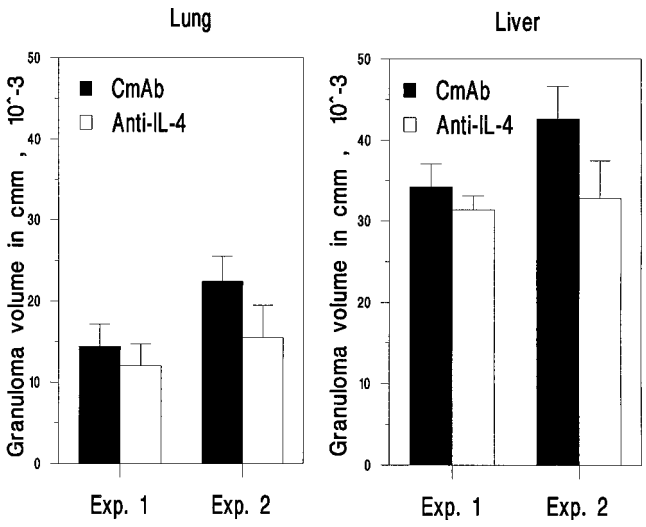

FIG. 2. Anti-IL-4 does not significantly reduce the granuloma size around laid eggs. Lung and liver granulomas were produced during infection by creating a porto-systemic shunt. Anti-IL-4 treatment had no significant effect on granulomas forming around eggs produced during infection. cmm, cubic millimiters; vertical bars, 1 standard error of the mean. No significant differences were seen between animals treated with anti-IL-4 and those treated with control MAb (CmAb).

was purified from ascites by ammonium sulfate precipitation. Egg-injected animals were given 1 mg of anti-IL-4 on day 0, 5 mg on day 1, and 2.5 mg on days 3, 6, and 10 after injection of the eggs (high-dose regimen). A dose of 1 mg of anti-IL-4 on days 0, 1, 3, and 10 (low-dose regimen) was also effective in nonsensitized mice. Infected animals were given 10 mg of anti-IL-4 at 4 weeks and 5 mg at 5, 6, and 7 weeks of infection. GL113 was given for each group in a similar regimen. To assess adequacy of treatment, IgE was measured by enzyme-linked immunosorbent assay (7).

Histology. For histologic evaluation, the lungs, after inflation with fixative, and livers were fixed in Bouin-Hollande solution and processed for routine paraffin embedding. The diameter of the granulomas was determined with an ocular micrometer in 4-μm sections stained with Litt's modification of Dominici's stain, and the volume was calculated for each granuloma by assuming a spherical shape. Slides stained with picrosirius red were examined in polarized light to evaluate fibrosis in the granulomas.

Cytokine measurement: isolation of RNA. RNase-free water and plastic were used throughout. The upper lobe of the right lung or 50 mg of liver was homogenized in 1 ml of RNA STAT-60 (Tel-Test "B," Inc., Friendswood, Tex.) with a tissue polytron (Omni International, Waterbury, Conn.), and total RNA was isolated as recommended by the manufacturer. The RNA was resuspended in diethylpyrocarbonate-treated water containing 1 mM EDTA and quantitated spectrophotometrically.

Reverse transcriptase PCR detection of cytokine mRNAs. A reverse transcription procedure was performed to determine the relative quantities of mRNA for IL-4, IL-5, and hypoxanthine phosphoribosyltransferase as previously described (20). In brief, after reverse transcriptase reaction of 1 μg of RNA, 10 μl of a 1:8 dilution of cDNA was amplified by PCR as previously described for each cytokine (20). Both positive and negative controls were included in the assay to confirm that only cDNA PCR products were detected and that none of the reagents were contaminated.

Quantitation of PCR products. After the appropriate number of PCR cycles, the amplified DNA was analyzed by electrophoresis, Southern blotting, and hybridization with nonradioactive cytokine-specific probes and quantified as previously described (20). The fold increase was calculated as the reciprocal of the equivalent dilution of the control cDNA (from uninjected and uninfected mice), and results were normalized by comparison with hypoxanthine phosphoribosyltransferase results when necessary.

Statistics. Student's *t* test was used to compare different groups. Results were considered significant when P was <0.05.

RESULTS

Anti-IL-4 treatment reduces granuloma size around i.v. injected *S. mansoni* eggs in both the liver and the lung. To see the effect of anti-IL-4 MAb on synchronous granulomas forming around injected eggs in the lung and liver, we injected the same animal with eggs in both the pulmonary and portal circulation on the same day. Anti-IL-4 treatment significantly reduced synchronous granulomas around injected eggs in both sites (Fig. 1). Granulomas in both sites were smaller in treated animals at 10 and 14 days after injection (Fig. 1, upper panels). The effects of anti-IL-4 were seen at both low and high doses of anti-IL-4 (Fig. 1, lower panels).

Anti-IL-4 does not significantly reduce granuloma size around eggs produced during infection in either the liver or the lungs. To see the effect of anti-IL-4 MAb treatment on granulomas around laid eggs in both the lung and the liver, we made a porto-systemic shunt to divert approximately half of the laid eggs to the lungs of infected mice. Anti-IL-4 treatment, begun before egg laying, had a small and insignificant ($P > 0.05$) effect on granuloma size around laid eggs in either site (Fig. 2), although it blocked IgE formation. Mean IgE levels in unexposed and control antibody- and anti-IL-4-treated animals were 2.33, 8.36, and 0.72 μg/ml, respectively ($P < 0.05$).

Anti-IL-4 reduces the size of granulomas around injected eggs in sensitized animals. To see whether the lack of effect of anti-IL-4 on granulomas around laid eggs seen in the previous experiments was due to prior sensitization of infected animals, we sensitized mice with eggs intraperitoneally and then challenged them 4 weeks later with 5,000 eggs i.v. In this case,

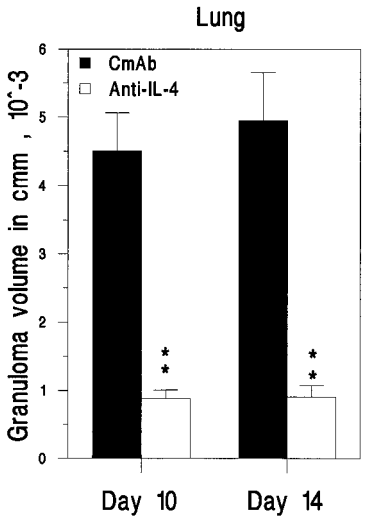

FIG. 3. Anti-IL-4 reduces granuloma size in sensitized animals. Four weeks after intraperitoneal sensitization, mice were injected i.v. with 5,000 eggs. Anti-IL-4 treatment significantly reduced granuloma volume. cmm, cubic millimeters; vertical bars, 1 standard error of the mean; **, $P < 0.01$ (compared with results in mice treated with control MAb [CmAb]).

anti-IL-4 treatment in the same dose used for infected mice significantly reduced granuloma size in sensitized animals (Fig. 3). Anti-IL-4 also reduced lung granuloma size around i.v. injected eggs in animals sensitized by experimental infection (4).

Granulomas around laid eggs were larger than those around injected eggs (4), and granulomas around both injected and laid eggs in the liver were larger than those in the lung (Fig. 1, 2, and 3). Fibrosis was also much more marked in the liver than in the lung, while arteritis was prominent in the lung. Animals treated with anti-IL-4, whether injected with eggs or infected, showed less fibrosis of the granulomas in both the liver and lung in picrosirius-stained sections (not shown), and in the liver fibrosis was also found to be diminished by quantitative determination of hydroxyproline levels (4). The granulomatous reactions around injected eggs in anti-IL-4-treated animals showed fewer epithelioid cells and eosinophils, as well.

IL-4 and IL-5 genes are prominently expressed in granulomas regardless of site or type of eggs. To assess the variation in cytokine patterns, we used a sensitive reverse transcriptase PCR technique that detects steady-state mRNA expression. Anti-IL-4 reduced Th2 cytokine mRNAs (IL-4 and IL-5) in the lung and the liver. It reduced these cytokines around laid as well as injected eggs (Fig. 4). Levels of mRNA for both IL-4 and IL-5 were consistently higher for tissues containing laid as opposed to injected eggs.

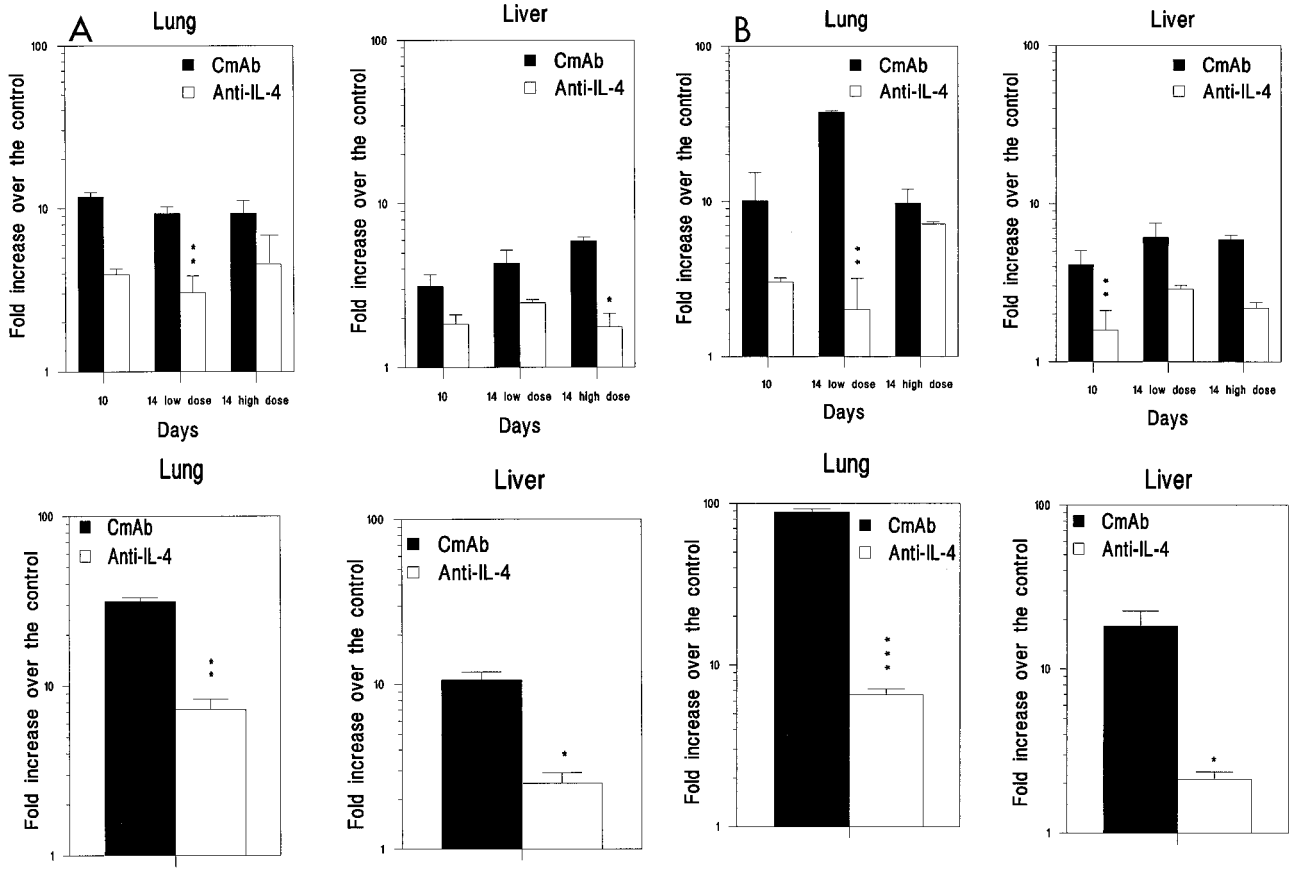

FIG. 4. Anti-IL-4 treatment reduces Th2 cytokine (IL-4 and IL-5) levels in both the lung and the liver and in infected as well as injected animals. (A) IL-4 mRNA steady-state profile; (B) IL-5 mRNA steady-state profile. (Top) Injected eggs; (bottom) laid eggs. Vertical bars indicate 1 standard error of the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared with results in animals injected with control MAb [CmAb]).

DISCUSSION

Since its first description, the model of synchronous pulmonary granulomas of *S. mansoni* was suggested to be not completely analogous to experimental infection (16, 17); however, it remains a useful tool to study the immunopathology of schistosomiasis. Recent data demonstrated that anti-IL-2 and anti-IL-4 (11, 20) reduced and anti-gamma interferon (11, 21) increased granuloma size in the lung model. Nevertheless, some of these antibodies have only a moderate (22) or minimal (2, 4, 15) effect on the size of hepatic granulomas forming during experimental infection. Additionally, recombinant IL-12 markedly decreased synchronous pulmonary granulomas (21) but had little effect on granuloma formation in infected animals (14a).

In this study, we have shown that the difference between the lung model and infection is most likely caused by differences between laid and injected eggs. The site of granuloma formation is not an adequate explanation for the differences observed between laid and injected egg-induced granulomas, because anti-IL-4 dramatically reduced the size of synchronous granulomas in both the liver and the lung and had little effect during infection at either site. Sensitization by prior exposure to egg antigens and nonspecific augmentation by adult worms during infection are also not convincing explanations for the observed differences between laid and injected eggs, because granulomas forming around injected eggs were reduced by anti-IL-4 treatment in animals sensitized by intraperitoneal eggs (Fig. 3) or by infection (4). The dose and regimen of anti-IL-4 MAb used were effective, since they blocked IgE production and reduced fibrosis associated with granuloma formation; however, it may be that the more antigenic eggs laid in vivo by the worms require less residual IL-4 for optimal granuloma formation than do the injected eggs.

The lower quality of injected eggs seems the most plausible explanation for the differences observed between laid and injected eggs. Thus, the injected eggs have already aged in the host and have been further altered by the isolation process. Eggs of *S. mansoni* gradually die (6, 12) and release their antigens in vivo (17) and, if maintained in culture for 14 days, lose their granulomatogenicity (9). von Lichtenberg noted that granuloma size and the amount of stainable egg antigens were greater around laid eggs than around injected eggs and even less in autoclaved eggs (17). In a related model, Hirata et al. found that larger granulomas formed around laid than injected eggs of *Schistosoma japonicum* (10). They also observed that the size of granuloma was independent of the nature of the host from which eggs were isolated or the presence of host immunoglobulin coating the eggs (10).

For the last 30 years, the synchronous granuloma model has been a very useful tool for understanding the immunopathology of schistosomiasis (1, 9, 18, 20, 21). Occasions in which the model clarified the immune response during patent infection are many: the role of different cells (13) and hypersensitivity reactions (18) in granuloma formation and the phenomenon of immune modulation (1) are but a few examples; however, differences between this model and natural infection exist. For instance, as seen in this study, granulomas tend to be larger and difficult to suppress by anti-IL-4 treatment in the latter. Lung granulomas tend to be smaller and resolve faster than liver granulomas. This may be related to a difference in oxygen tension (6); however, cellular composition (6, 14, 19) and cytokine profiles, as seen in this study, are similar in both granulomas.

Thus, the same stimuli appear important for the induction of granulomas in infected mice and in the lung model, but the lung model is more sensitive to down- and up-regulation. Interestingly, although anti-IL-2 and anti-IL-4 do not markedly down-regulate granuloma size in infected mice, these treatments do markedly decrease fibrosis in the granulomas (4), an effect paralleled by the decrease in granuloma size in the lung model.

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