Tolerization of Mice to *Schistosoma mansoni* Egg Antigens Causes Elevated Type 1 and Diminished Type 2 Cytokine Responses and Increased Mortality in Acute Infection

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Tolerization of Mice to Schistosoma mansoni Egg Antigens Causes Elevated Type 1 and Diminished Type 2 Cytokine Responses and Increased Mortality in Acute Infection

Padraic G. Fallon and David W. Dunne

The granuloma that surrounds the Schistosoma mansoni egg is the cause of pathology in murine schistosomiasis, and its formation is driven by egg Ag-stimulated type 1 and type 2 cytokines. To determine the role of egg-driven immune responses during schistosome infection we rendered CBA/Ca mice unresponsive to schistosome eggs by combined cyclophosphamide treatment and thymectomy. In the early acute stages of schistosome infection, egg-tolerized mice suffered high mortalities. Granuloma size and deposition of collagen in the liver were significantly reduced in egg-tolerized mice. Similarly, limited granuloma responses were detected in the intestines of these mice, and this was associated with a >90% reduction in egg excretion. Histologically, egg-tolerized mice had exacerbated hepatocyte damage, with extensive microvesicular steatosis. Elevated plasma transaminase levels confirmed the damage to hepatocytes. Infected egg-tolerized mice had impaired proliferation responses to egg Ag but intact responses to worm Ag. Tolerized mice had diminished Ab responses to egg Ag and had a type 1 cytokine isotype pattern to worm Ag, with elevated IgG2a and diminished IgG1 and IgE. Egg-tolerized mice failed to down-regulate type 1 cytokines that are normally elicited during early schistosome infection. Hepatic granuloma cells from egg-tolerized mice were also type 1 cytokine dominated, with elevated frequencies of Tc1/Th1 and reduced Tc2/Th2 cells. This study demonstrates that mice tolerized to schistosome eggs have elevated type 1 cytokine responses with diminished type 2 responses and reduced anti-egg Ab during schistosome infection, and these effects are detrimental to the host. The Journal of Immunology, 1999, 162: 4122–4132.
was associated with type 1 cytokine-dominated responses and diminished type 2 cytokines. These studies show that egg-stimulated type 2 cytokine responses during schistosome infection of mice may regulate proinflammatory type 1 cytokine responses elicited by the other stages of the parasite life cycle.

Materials and Methods

Parasite, mice, and Ag preparations

A Puerto Rican strain of S. mansoni was used in all experiments. The parasite was maintained in laboratory passage in Biomphalaria glabrata snails and outbred Tyler’s Original mice. Female CBA/Ca mice, obtained from Harlan (Bicester, U.K.), were used for all experiments. Mice were maintained under specific pathogen-free conditions. Isolation of schistosome eggs from the livers of infected mice and the preparation of soluble egg Ag (SEA) and adult worm Ag (AW) were previously described (8). OVA was obtained commercially (Sigma, Dorset, U.K.).

Tolerization protocol

Thymectomy was performed on adult mice the day before egg injection. Mice were injected i.p. with 10,000 S. mansoni eggs. The day after egg injection Cγ (Sigma) was administered i.p. (200 mg/kg) in divided doses for 4 consecutive days. Four weeks after treatment mice were used in experiments. All animals were placed on antibiotic-supplemented water.

Parasitological techniques

In preliminary studies egg-tolerized mice that were exposed to 120 S. mansoni cercariae had high mortalities. Therefore, the data presented in this paper are from mice that were percutaneously infected with 60 S. mansoni cercariae. Infection and portal perfusion were performed as described previously (15). The liver and intestine were removed and used for tissue egg counts. Tissues were digested in 4% KOH as previously described (16). Fecal samples were collected on the day before termination, and eggs were counted (16). In accordance with our U.K. Home Office regulations, any infected mice that became moribund were humanely killed.

Pathological measurements

To quantitatively hepatocyte damage, plasma samples were collected from mice during infection or on the day of termination for transaminase assay. Glutamic oxalacetic transaminase (GOT) levels were determined using a commercial kit (Sigma); GOT levels are expressed as Sigma Frankel units per milliliter. The two ventral median lobes of the liver were fixed in formal saline and embedded in wax, and 5-μm sections were cut. Liver sections were stained with hematoxylin and eosin for granuloma diameter measurement and hepatotoxicity scoring or were stained with Martius Scarlet Blue for examination of fibrosis in the liver. Tissue collagen in the liver was quantified by differential staining of sections (three per mouse) on slides (17). Liver sections were deparaffinized and incubated with a saturated solution of picric acid in distilled water containing 0.1% Fast Green FCF (which stains noncollagenous proteins) and 0.1% Sirius Red F3B (which stains collagen). After 2-h incubation in the dark at room temperature, the slides were rinsed in distilled water until the elution fluid was completely free of color. Each slide was covered in 1 ml of 0.1 N NaOH in absolute methanol (1:1, v/v) for a few seconds until all the color was eluted from the section. The fluid on the sections was carefully transferred to cuvettes, and the absorbance was read at 605 nm (maximal absorbance of Fast Green) and 540 nm (maximal absorbance of Sirius red) on a spectrophotometer. The amount of collagen, expressed as micrograms collagen per milligrams of protein, was calculated using the formula described previously (17). For each mouse the diameters of 21 individual egg granulomas were measured, and livers from 10–15 mice were examined per group (minimum of 210 each mouse the diameters of 21 individual egg granulomas were measured, and single cell suspensions were obtained by passing cells through 70-μm cell strainers (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). The RBC were lysed in Tris-ammonium chloride solution. RPMI 1640 (Sigma) supplemented with 50 μM penicillin and 50 μg/ml streptomycin (Life Technologies, Paisley, Scotland) was used to wash cells. Cells were stained with trypan blue, and viable cells were counted. The cell concentrations were adjusted to 5 × 10⁶ cells/ml in culture medium (RPMI 1640-Dutch Modification; supplemented with 10% FCS (Sigma), penicillin-streptomycin (50 μg/ml to 50 μg/ml), 2-ME (50 μM), and glutamine (2 mM; Life Technologies)). A final cell concentration of 5 × 10⁶ cells/ml was added to a 24-well microtiter plate (Flow, McLean, VA). Cells were stimulated with 10 μg/ml SEA, AW, or anti-CD3 (25 μg/ml; PharMingen, San Diego, CA). Supernatants were collected after 24 and 72 h of culture and were stored at −20°C. TNF-α assays were performed immediately on supernatants collected from 24-h cultures.

For proliferation assays cells were plated (5 × 10⁶/well) on 96-well microplates. Triplicate wells were stimulated with different quantities of Ag (SEA, AW, or OVA) and cultured for 96 h. One microcurie of [3H]thymidine (Amersham, Aylesbury, U.K.) was added for the last 18 h of culture. [3H]thymidine incorporation was monitored and expressed as counts per minute.

Granulomas were isolated using methods modified from previously described protocols (19, 20). Briefly, infected mice were perfunctorily perfused with cold RPMI 1640 with heparin added. The entire liver was removed and homogenized using a blender (MSE Scientific, Sussex, U.K.). Intact granulomas were isolated by sedimentation (300 × g) and three washes in RPMI 1640. Granulomas were disrupted by incubation in RPMI 1640 with 1500 U/ml collagenase (type IV from Clostridium histolyticum; Sigma) for 35 min at 37°C with constant stirring. After incubation the solution was passed through a 5-ml syringe to disperse the digested granulomas. The granuloma suspension was sieved through 70-μm pore size cell strainers (Falcon, Becton Dickinson, Franklin Lakes, NJ) to remove nondispersed fragments. Granuloma cells were collected after repeated centrifugation and washing in RPMI 1640. Cell viability was determined to be >95% by trypan blue staining and flow cytometry after propidium iodide staining. Granuloma cells were cell phenotyped and cultured with anti-CD3 for 8 h (intracellular cytokine analysis) or for 24 h for cytokine assays on supernatants.

Cytokine ELISA

The levels of IL-2, IFN-γ, IL-4, IL-5, IL-10, IL-12, TGF-β1, and TNF-α in cell culture supernatants or serum were quantified using ELISA. For the assay of IL-2, IFN-γ, IL-4, IL-5, and IL-10, anti-cytokine monoclonals and recombinant cytokine standards were purchased from PharMingen (San Diego, CA) or Genzyme (Kent, U.K.). Immulon II plates (Dynatech, Chantilly, VA) were coated with 50 μl/well of capture anti-cytokine monoclonal (1–3 μg/ml) in 0.1 M NaHPO₄, pH 9.0 overnight at 4°C. PBS-T (PBS with 0.05% Tween-20) was used to wash plates, and they were blocked for 1 h in 1% BSA in PBS-T. Serial dilutions of recombinant cytokine or cell supernatants were added (50 μl/well) for 2 h at room temperature. Biotinylated anti-cytokine detection mAb (0.5–2 μg/ml) were added for 2 h at room temperature. ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) was used as substrate, and plates were read at 405 nm. The concentrations of IL-2 (picograms per milliliter), IL-4 (units per milliliter), IL-5 (nanograms per milliliter), IL-10 (units per milliliter), and IFN-γ (nanograms per milliliter) were interpolated from the appropriate recombinant cytokine standard curve. TNF-α was quantified using a commercial ELISA kit according to the manufacturer’s instructions (Genzyme, Cambridge, MA). Total TGF-β1 (acidified samples) was measured with a commercial assay according to the manufacturer’s instructions (Promega, Southampton, U.K.). IL-12 was measured with paired mAb and recombinant cytokine that were gifts from Cambridge Bioscience (Cambridge, U.K.).

Flow cytometry and intracellular cytokine detection

Intracellular cytokine staining was used to determine the frequency of IL-4- or IFN-γ-producing CD4⁺ and CD8⁺ T cells as previously described (8). All reagents for intracellular cytokine staining were purchased from Calbiochem (Burlingame, CA). Cells were cultured at 10⁶/ml in 24-well plates (Becton Dickinson) for 8 h in the presence of medium alone or with plate-bound anti-CD3 (25 μg/ml; PharMingen). Brefeldin A (5 μg/ml; Sigma) was added for the last 3 h of culture. Cells (10⁶/well) were transferred to 96-well U-bottomed microtiter plates (Dynatech). Cells were blocked for 10
min on ice in 50% FCS in FACS buffer (PBS/1% FCS). TRI-Color-conjugated anti-mouse CD4 or CD8 was added for 45 min on ice to phenotype cells. After washing, cells were fixed in fixation medium (CalTag). Permeabilization of cells occurred in the presence of FITC-conjugated anti-mouse IFN-γ and phycoerythrin-conjugated anti-mouse IL-4. After washing, the plates were stored overnight at 4°C in FACS fixative buffer (FACS buffer with 1% paraformaldehyde). Flow cytometry was performed on a FACScan with LYSIS II software (Becton Dickinson Immunocytometry Systems, San Jose, CA). In all experiments unstained cells and cells stained separately with each fluorochrome were included to optimize compensation settings. The addition of excess recombinant cytokines or unlabeled anti-IL-4 or IFN-γ Ab was used to confirm that the cytokine signals detected were specific. Lymphocytes were gated on their forward and side scatter characteristics. TRI-Color (FL3)-stained CD4 or CD8 lymphocytes were gated, and 15,000 events were analyzed per sample. The percentage of CD4+ or CD8+ T cells containing intracytoplasmic IL-4 or IFN-γ in appropriate quadrants was determined. Three replicates of stained cells were FACS acquired/analyzed separately in each experiment, and the mean positive cells of the triplicate determinations was calculated. Results are presented as the mean percentage of positive cells obtained from at least three different experiments. Cells from the spleen or granulomas of individual mice were phenotyped with fluorochrome-labeled anti-CD3, anti-CD4, anti-CD8, and B220.

ELISA for Ag-specific Ab responses
Sera were collected from mice before infection and on the day of termination. IgG, IgG1, IgG2a, and IgE isotype responses to AW, SEA, and OVA were evaluated using an ELISA. Optimum conditions for ELISA were obtained by checkerboard titrations with pooled positive (from 20-wk-infected mice) and negative (uninfected mice) serum. Microtiter plates were coated overnight with Ag. After three washes in PBS-T, plates were blocked with 1% BSA. Individual mouse serum (1/200 for IgG, IgG1, and IgG2a and 1/25 for IgE) was added in triplicate wells. IgG, IgG1, and IgG2a were detected with horseradish peroxidase-conjugated anti-mouse isotype Ab (Sigma or Zymed (San Francisco, CA)). For detection of IgE, plates were incubated with biotinylated anti-mouse IgE followed by streptavidin-peroxidase. Three separate experiments were analyzed; in each experiment 10–15 mice were used in each group.

Statistical analysis
Statistical differences between groups were determined using Student’s t test. A p value of <0.05 was considered significant.

Results
Induction of tolerance of mice to schistosome eggs
Administration of Cy with Ag induces a state of Ag-specific tolerance (21). We used Cy treatment to induce tolerance of mice to schistosome eggs. Mice were injected with schistosome eggs and treated with Cy; control groups included mice injected with eggs alone or mice treated with Cy alone. Four weeks after treatment mice were injected with 5000 eggs into the hind footpads, and 14 days later the draining PLN were removed. Cells from the PLN were restimulated in vitro with varying quantities of SEA, and proliferation responses were determined by [3H]thymidine incorporation. The Cy(+) egg-injected mice had substantially impaired proliferation responses to SEA compared with the responsiveness of the other groups (Fig. 1A). To test whether the responses to all Ag were impaired in the Cy(+) egg-treated mice, OVA was injected into the footpad, and proliferation responses to OVA were assayed. The Cy(+) egg-treated group had similar proliferation responses to OVA as control groups (Fig. 1B). As humoral responses are also modified by Cy-induced tolerance, we measured Ab responses to SEA or OVA. Cy(+) egg-treated mice had impaired Ab responses to SEA, but mounted normal Ab responses to OVA (not shown). The treatment of mice with Cy after injection with S. mansoni eggs induces a state of Ag specific tolerance to parasite egg Ag.

Peripheral tolerance to Ag induced by various methods may wane after tolerance induction, with mice having the capacity to partially restore responsiveness as early as 5 wk after induction of tolerance by Cy treatment (22). As we wanted to study acute (6–8 wk) and chronic (14–16 wk) stages of infection, a concern was that the emergence of responsive nontolerized cell populations may have compromised any results obtained. Mice were treated with Cy(+) egg, and at various times after tolerance induction they were examined for responsiveness to egg Ag. Four weeks after treatment tolerantized mice were unresponsive to SEA, but by 8 wk after the induction of tolerance the responsiveness of tolerantized mice to SEA was partially restored (not shown). A commonly used strategy to circumvent waning of tolerance is to remove the thymus and prevent replenishment of peripheral T cells (22, 23). Mice were thymectomized before tolerance induction, and the unresponsiveness to challenge with eggs was tested. Tolerized mice that had been thymectomized remained unresponsive to egg Ag (proliferation responses and Ab production) for at least 20 wk after treatment (not shown). The longevity of the unresponsiveness induced to schistosome eggs is prolonged by removal of the thymus. Therefore, in all subsequent experiments thymectomy was included as part of the tolerization protocol.

Effects of egg tolerization on schistosome infection
During initial studies egg-tolerized animals were infected with 120 S. mansoni cercariae. By day 50 after infection there were high mortalities (>90%) of the egg-tolerized mice, with no deaths by this stage of infection in any control animals. On the basis of these results additional experiments were performed using a lighter infection, with mice exposed to 60 cercariae. In three separate experiments following exposure of mice to 60 cercariae there was high mortality (>80%) of egg-tolerized mice by the eighth week of schistosome infection, whereas <5% of mice in thymectomized, Cy-treated, egg-injected or combined thymectomy and Cy-treated control groups had died. As shown in Fig. 2, mice were
dying from day 45 after infection, i.e., within approximately 14 days of the onset of egg laying; by the 51st day after infection >50% of the egg-tolerized mice had died, with these deaths occurring within a period of 6 days (days 45–51). Egg-tolerized mice with lighter infections (exposure to 25 cercariae) also suffered high mortalities (>70%) by the 8–9th week of infection. In all cases, regardless of the level of parasite infection, death of egg-tolerized mice occurred during the acute stages of infection. In all experiments low mortalities (<5%) were observed in all control (thymectomized, Cy-treated, egg-injected, or combined thymectomy and Cy-treated) groups. The early mortality of egg-tolerized mice during infection prevented studies during the chronic stage of infection. We therefore focused on pathology and immune responses in egg-tolerized mice during the early acute (7th week) stages of infection.

S. mansoni-infected egg-tolerized animals had no statistical difference in worm recovery (number of worm pairs) and fecundity (eggs in the liver or intestine) than control groups (Table I). These data indicated that the death of egg-tolerized mice was not due to any direct effect of treatment on the parasite. Egg-tolerized mice had dramatically reduced egg excretion, with >90% fewer eggs being excreted in the feces of tolerized mice compared with control animals (Table I). The inability of egg-tolerized animals to excrete eggs in the feces was partially reflected in marginal higher levels of eggs present in the intestine of these mice compared with those in control infected mice (Table I). As the liver is a major organ affected during schistosome infection of mice we studied the pathology in this organ. Macroscopically, the livers from egg-tolerized mice had a whitened fatty appearance compared with livers from normal infected mice (Fig. 3A). The sizes of granulomas surrounding eggs in the livers of tolerized mice were significantly smaller (p < 0.001) than those of granulomas in control infected animals (Table II and Fig. 3, B and C). Similarly, we have observed that pulmonary granuloma after i.v. injections of eggs are diminished in size in egg-tolerized animals (not shown). We also measured collagen within the liver, as fibrosis is a sequel to egg granuloma formation during schistosomiasis. Egg-tolerized mice had a significant reduction in collagen deposition in liver tissue (Table II). Martius Scarlet Blue staining visually demonstrated the dramatically smaller granulomas surrounding the schistosome egg and also the reduced amount of collagenous material (blue stain) in the liver of egg-tolerized animals compared with those in control animals (Fig. 3, B and C). Sections of liver from egg-tolerized mice showed extensive hepatocyte damage; this cell damage (e.g., loss of cytoplasmic staining in hepatocytes) was external to the granuloma and was dispersed in the liver parenchyma (Fig. 3E). Higher magnification revealed ballooning degeneration and microvesicular steatosis of hepatocytes in egg-tolerized animals (Fig. 3G). There was limited hepatocyte damage in infected control animals (Fig. 3, D and F). Using a previously defined arbitrary scale to quantify hepatocyte damage in schistosome-infected mice (18), double-blind scoring of liver sections confirmed the extensive hepatocyte damage in the egg-tolerized mice (Table II). We also measured the plasma levels of GOT, as plasma transaminases are markers for hepatocyte damage. Infected egg-tolerized mice, had significantly elevated levels of GOT (p < 0.001) compared with control infected animals (Table II). These effects on the liver were not directly attributable to an increase in the numbers of schistosome eggs in the livers of egg-tolerized mice, as they had comparable numbers of eggs in the liver as control animals that had limited liver damage (Table I). When we studied the livers of uninfected animals there was no difference (liver collagen, hepatocyte integrity, or plasma GOT levels) between egg-tolerized or control mice. Collectively, these data demonstrated that following schistosome infection the livers of egg-tolerized mice had severe pathology that is associated with extensive hepatotoxicity.

As egg-tolerized mice had almost completely lost the ability to excrete eggs in their feces (Table I), we also examined the guts of these animals. Tissue digests of liver or guts from infected mice showed that there was no significant difference in the relative distribution of eggs in the liver or gut between egg-tolerized and control groups (Table I); this indicates that the failure of egg-tolerized mice to excrete eggs is not due to aberrant egg deposition by the parasite. Histologically, the intestines of infected normal mice were characterized by the appearance of granulomas surrounding eggs in the submucosa, with eggs also detected within granulomas in the lamina propria and along the villi, and with eggs in various stages of extravasation to the lumen (Fig. 3H). In contrast, in all sections of intestines from egg-tolerized mice, eggs were predominately detected in the submucosa, with few eggs detected in the lamina propria or ascending the villi. No or limited granulomatous responses were apparent in the vicinity of eggs in the intestine of egg-tolerized mice (Fig. 3I). Similar to the hepatic granuloma, there were fewer eosiophils detected in the vicinity of the egg in the intestine. An additional observation was that there was significantly more (p < 0.001) collagen in the intestines of infected egg-tolerized mice (12.6 ± 1.28 μg/mg; mean of 10 infected mice) compared with that in control infected mice (10.6 ± 0.8 μg/mg; mean of nine mice). However, the levels of collagen in uninfected egg-tolerized mice were also significantly higher (p < 0.01) than those in uninfected control animals (not shown). This

![FIGURE 2. Survival of S. mansoni-infected normal and egg-tolerized mice.](#)

<table>
<thead>
<tr>
<th>Tissue Eggs</th>
<th><strong>Worm Pairs</strong></th>
<th>Liver</th>
<th>Intestine</th>
<th>Faecal Eggs (eggs/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7.5 ± 1.5</td>
<td>12,137 ± 386</td>
<td>9,849 ± 154</td>
<td>212 ± 75</td>
</tr>
<tr>
<td>Egg-tolerized</td>
<td>6.8 ± 2.1</td>
<td>11,921 ± 249</td>
<td>10,085 ± 279</td>
<td>11 ± 43</td>
</tr>
</tbody>
</table>

* Mice were percutaneously infected with 60 S. mansoni cercariae. Mice were terminated 49 days after infection, and worm burden and tissue egg counts were performed as described in Materials and Methods. Data are presented as group mean ± SD, with 12 mice per group. In four separate experiments there were no differences in parasite infectivity (number of worm pairs) or egg distribution (tissue egg counts) between egg-tolerized mice and any simultaneously infected control groups of animals.

* In all experiments egg-tolerized mice had significantly less eggs in their faeces compared to control groups; p < 0.001 by Student’s t test.
increase in intestinal collagen in egg-tolerized mice is due to the Cy treatment, as mice treated with Cy also had increased intestinal collagen. However, mice that were only treated with Cy had normal egg excretion during schistosome infection.

**Cellular and humoral responses in schistosome-infected egg-tolerized mice**

Spleen cells from thymectomized, Cy-treated, egg-injected, combined thymectomized and Cy-treated, or Cy-treated, thymectomized, and egg-injected mice were analyzed by flow cytometry to ascertain whether the treatment protocol affected CD4<sup>+</sup>, CD8<sup>+</sup>, and B220<sup>+</sup> cell populations. The combined thymectomy and Cy treatment, with or without concurrent egg injection, induced a transient lymphopenia in mice, with a 20–50% reduction in CD3<sup>+</sup> or B220<sup>+</sup> cells in the spleen detected 24 h after the last Cy treatment. By the day of infection (4 wk after Cy treatment) spleen lymphocyte numbers (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, B220<sup>+</sup>) had returned to pre-treatment levels. This recovery of lymphocyte populations after

### Table II. Liver pathology in S. mansoni-infected normal and egg-tolerized mice<sup>a</sup>

<table>
<thead>
<tr>
<th>Group</th>
<th>Granuloma Diameter (µm)</th>
<th>Collagen (µg collagen/mg protein)</th>
<th>Hepatotoxicity (arbitrary units)</th>
<th>Plasma GOT (SF U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>329 ± 22</td>
<td>13.6 ± 0.8</td>
<td>0.07 ± 0.05</td>
<td>42.3 ± 5</td>
</tr>
<tr>
<td>Egg-tolerized</td>
<td>219 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.3 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.1 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>142.1 ± 14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are presented as mean ± SD and are representative of results obtained from three different experiments. Ten to 15 individual animals were used per group in each experiment. Parameters were quantified as described in Materials and Methods.

<sup>b</sup>A p value of <0.001 shows significant difference between groups. Statistical analysis was performed by Student’s t test.
combined Cy treatment and thymectomy of adult mice has been reported previously (24). Spleens and MLNs were removed from mice during the seventh week of schistosome infection. Spleen cells from infected egg-tolerized mice were restimulated in vitro with AW and SEA to determine whether proliferation responses to parasite Ag were impaired after infection. Cells from egg-tolerized mice had impaired proliferation responses when restimulated with a range of concentrations of SEA, whereas control infected animals were responsive to SEA (Fig. 1C). However, consistent with the tolerance being egg Ag specific, egg-tolerized mice had unimpaired proliferation responses to worm Ag, with AW responses comparable to those in control animals (Fig. 1D).

To evaluate what cytokine responses were elicited during schistosome infection, cells from infected mice were cultured in vitro in the presence of parasite Ag (AW or SEA) or were stimulated with anti-CD3. ELISA of the supernatants from cultures demonstrated that when stimulated with anti-CD3, cells from control infected animals produced predominately type 2 cytokines (Fig. 4). In contrast, anti-CD3-stimulated cells from egg-tolerized mice produced significantly more type 1 cytokine (IL-2, IFN-γ, and TNF-α; Fig. 4, A–C) and significantly less type 2 cytokine (IL-4, IL-5, and IL-10; Fig. 4, D–F) than control animals. SEA, a known stimulator of type 2 cytokines during schistosome infection, elicited the secretion of IL-4, IL-5, and IL-10 in control animals, but, consistent with the cell proliferation data, this Ag preparation stimulated limited cytokine secretion by cells from egg-tolerized mice. Restimulation of cells from infected egg-tolerized mice with AW caused the abundant production of IFN-γ (four- to fivefold more produced than in cells from control infected animals), IL-2 (two- to threefold higher levels), and TNF-α (two- to threefold greater levels); with AW not stimulating the secretion of type 2 cytokines. Similar cellular responses were observed in MLN cells. These data reveal that by the seventh week of schistosome infection egg-tolerized mice have a type 1 cytokine-dominated response that is also associated with diminished type 2 cytokines, with AW stimulating type 1 cytokine secretion by cells from egg-tolerized mice. In the context of the type 1/type 2 cytokine dichotomy the cytokine profile of infected egg-tolerized mice is almost the complete opposite of that of the type 2 cytokine-dominated response observed in normal infected mice.

Serum recovered from infected mice was tested in ELISA for Ag-specific Ab responses. Total IgG, IgG1, IgG2a, and IgE responses of egg-tolerized mice to worm (AW) and egg Ag (SEA) by S. mansoni-infected normal and egg-tolerized mice. The mean ± SD values from 12 mice/group are presented. Comparable results were obtained in three separate experiments (10–15 mice/group). *** p < 0.001, significant difference between groups. Statistical analysis was performed using Student’s t test.
may reflect the worm Ag-driven type 1 cytokine environment (Fig. 4) of these animals.

Failure of egg-tolerized mice to down-modulate type 1 responses during schistosome infection

Characteristically, during the course of schistosome infection of mice there is a gradual switch from a predominant type 1 cytokine response during the first 4 wk of infection to a type 2 cytokine-dominated response after egg laying (6, 8). However, as we have shown above, egg-tolerized mice have a type 1 cytokine-dominated response with diminished type 2 cytokines by the seventh week of infection. Thus, we investigated whether this type 1 cytokine bias occurred earlier in infection. Spleen cells were removed from infected mice on day 28 (before egg laying; when egg-tolerized mice had a type 1 cytokine-driven response) and day 47 after infection. Anti-CD3 treatment stimulated marginally more Th1 or Th1 cells in tolerized mice compared with those in control animals by 28 days after infection (Fig. 6, D and E). In contrast to the marked reduction by day 47 postinfection in the frequency of Th1 or Th1 cells observed in normal mice, there was a limited decline in these cell populations in egg-tolerized mice. Egg-tolerized mice also did not have the expansion in the frequencies of Th2 cells that occurred in control animals later during infection (Fig. 6F).

A type 1 cytokine environment prevails within the liver granuloma of egg-tolerized mice

The cells that constitute the liver granuloma have been used previously to analyze the cytokine responses that intimately regulate formation of the egg granuloma (13, 20). To address the question of whether the generalized type 1 cytokine responses in egg-tolerized animals was reflected in granuloma cell populations, we isolated granuloma cells from the livers of mice from 6–7 wk after infection. Initial attempts using standard protocols (19, 20) to isolate cells from liver granulomas of egg-tolerized mice were confounded by the high levels (>50%) of propidium iodide-stained (dead) cells detected by flow cytometry, compared with <5% stained cells that were recovered from granulomas of normal mice. By reducing the amount of collagenase used to disrupt the intact granuloma, the levels of cell damage was reduced <5% in egg-tolerized mice, but this amount of enzyme failed to adequately separate granuloma cells from the livers of normal infected mice (the requirement for more collagenase treatment to isolate cells from the granulomas of infected mice compared with egg-tolerized mice is probably due to the greater amount of collagen in livers of normal mice compared with that in egg-tolerized mice; Table II). Therefore, by extending the collagenase digestion incubation time to 35 min, <5% damaged cells (propidium iodide) were recovered from liver granulomas from mice. Consistently, three- to sixfold fewer granuloma cells were recovered from the livers of individual egg-tolerized mice compared with cell recovery from normal infected mice; this reduction in granuloma cell recovery may be a reflection of the smaller sized granulomas in these animals (Table II).

ELISA detection of IL-4 and IFN-γ in the supernatants from 24-h cultures of anti-CD3-stimulated granuloma cells showed that there was a greater propensity for granuloma cells from egg-tolerized animals to secrete IFN-γ and, conversely, a diminished capacity to produce IL-4 compared with secretion of these cytokines by granuloma cells from normal infected mice (Fig. 7, A and B). Using intracellular cytokine staining it was shown that granuloma cells from normal infected mice had a mixed composition of marginally more production of this cytokine by cells from tolerized animals at this stage of infection (Fig. 6A). By day 47 of infection the cytokine responses of cells from egg-tolerized mice were strikingly different from responses of control animals; there was an increase in the secretion of IFN-γ and, conversely, no elevation in IL-4 secretion (Fig. 6, A and B). In normal animals the increase in IL-10 secretion was coincident with the reduction in IFN-γ release by cells (Fig. 6C). In egg-tolerized mice when we examined IL-10 secretion we observed limited production of this cytokine during infection (Figs. 4 and 6C). The absence of IL-10 and IL-4 in acute infection may be partially responsible for the inability of tolerized mice to down-regulate type 1 cytokine responses that are induced by larval or worm Ag early during schistosome infection.

To investigate whether differences between egg-tolerized and normal mice in type 1 and type 2 responses were due to modification in the frequencies of type 1 or type 2 cytokine secreting CD4+ or CD8+ cells, we performed intracellular cytokine staining and flow cytometry on MLN cells recovered 28 and 47 days after infection. Anti-CD3 treatment stimulated marginally more Tc1 or Th1 cells in tolerized mice compared with those in control animals by 28 days after infection (Fig. 6, D and E). In contrast to the marked reduction by day 47 postinfection in the frequency of Tc1 or Th1 cells observed in normal mice, there was a limited decline in these cell populations in egg-tolerized mice. Egg-tolerized mice also did not have the expansion in the frequencies of Th2 cells that occurred in control animals later during infection (Fig. 6F).

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IFN-γ- and IL-4-stained CD4 and CD8 cells; within CD8 cells Tc1 were more abundant, and conversely with respect to CD4+ cells there was approximately a 2:1 ratio of Th2 cells to Th1 (Fig. 7, C and D). There were negligible frequencies (<0.01%) of IL-4-stained CD4+ or CD8+ cells within granuloma cells recovered from egg-tolerized livers (Fig. 7, E and F). In contrast, there was a marked increase in the frequencies of Tc1 (>4% positive) and Th1 (>5% positive) cells within granuloma cell populations from egg-tolerized mice. The detection by flow cytometry of predominately IFN-γ-secreting CD4+ or CD8+ cells in granulomas of egg-tolerized mice suggests that in these animals there are increases in the frequencies of IFN-γ production by T cells within the egg granuloma and a reciprocal reduction in IL-4-secreting cells. 

The secretion of TNF-α and IL-12 by granuloma cells from egg-tolerized mice was considerably higher than secretion of these cytokines by granuloma cells from normal infected animals (Table III). The relatively reduced secretion of IL-10 and TGF-β1 by granuloma cells from egg-tolerized mice and the increased IL-12 levels may be driving the increased secretion of IFN-γ and TNF-α within the granuloma.

### Discussion

During infection of mice with *S. mansoni*, a hepatointestinal disease that is characterized by granulomatous inflammation to parasite eggs trapped in host tissue. In the context of type 1 and 2 cytokines it has been shown that there is an initial role for type 1 cytokines in early granuloma formation which subsequently is superseded by a type 2 dominated responses against the egg (27). To elucidate the influences of egg-elicited immune responses on schistosome pathology we induced a state of egg-specific immunological unresponsiveness (tolerance) in mice before schistosome infection. Egg-tolerized mice suffered severe mortality during the early acute stages (7–8 wk) of schistosome infection. This mortality was associated with elevated type 1 cytokines and diminished type 2 cytokines as well as impaired anti-egg Ab production. The method used in this study to induce specific unresponsiveness of mice to schistosome eggs is a novel model to elucidate the role of immune responses elicited by parasite Ag in the pathology of schistosomiasis.

In earlier studies, McCurley and colleagues (28) attempted to induce unresponsiveness to schistosome eggs using various protocols to induce tolerance, including intrathymic injection of SEA, oral administration of eggs or SEA, and high and low dose Ag injection. After i.v. administration of egg Ag there was an indication that following high dose injection a state of tolerance was induced, i.e., mice injected twice with 1 mg of SEA had smaller pulmonary granulomas than control PBS-injected animals. All other tolerance protocols attempted failed to cause unresponsiveness (28). Similarly, mice treated with SEA and Cy have been shown to have smaller pulmonary granulomas (29). These two studies used soluble egg Ag to induce unresponsiveness, whereas in this study we used whole eggs. We induced partial unresponsiveness to SEA using Cy treatment and also observed smaller pulmonary granulomas. However, following schistosome infection mice that are rendered unresponsive to SEA have normal hepatic granulomas (not shown). That responses that participate in the formation of the schistosome egg granulomas in the lungs and the liver may differ has also been observed previously in normal (30) and IL-4-deficient (31) mice.

Following schistosome infection of STAT6-deficient mice, pathology similar to that described here occurs (smaller granuloma and reduced hepatic collagen) with cytokine responses comparable to the data obtained here with egg-tolerized mice, i.e., a generalized skewing to a type 1 cytokine profile (14). Data from studies

### Table III. Production of TNF-α, IL-12, IL-10, and TGF-β1 by granuloma cells recovered from the livers of schistosome-infected normal or egg-tolerized mice

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α (pg/ml)</th>
<th>IL-12 (pg/ml)</th>
<th>IL-10 (U/ml)</th>
<th>TGF-β1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>124 ± 12</td>
<td>15 ± 4</td>
<td>35 ± 6</td>
<td>489 ± 43</td>
</tr>
<tr>
<td>Egg-tolerized</td>
<td>455 ± 85</td>
<td>147 ± 38</td>
<td>10 ± 3</td>
<td>98 ± 15</td>
</tr>
</tbody>
</table>

*Granuloma cells were isolated from the livers of individual *S. mansoni*-infected normal and egg-tolerized mice. Cells were cultured in triplicate for 24 h in the presence of anti-CD3. Cytokine levels present in supernatants were determined by ELISA. Values are mean ± SD of assays on supernatants collected from three separate mice per group. Data shown are representative of results obtained from four different experiments, with two to four individual livers from each group analyzed separately in each experiment.
on IL-4-deficient mice, which may be influenced by experimental conditions and the genetic background of the animal, have also shown that there is a modified pulmonary (naïve mice injected with eggs i.v.) (31) and hepatic granulomas (eggs deposited in situ) (32) in these animals. Excessive hepatic collagen deposition and the formation of fibrosis are causes of pathology during schistosome infection. In egg-tolerized mice there was a significant reduction in the amount of hepatic collagen. The elevated IFN-γ secretion by cells recovered from within the hepatic granuloma of egg-tolerized mice may be relevant, as IFN-γ can regulate collagen formation (33). Indeed, administration of recombinant IFN-γ to schistosome-infected mice has been shown to reduce collagen deposition (34, 35). The relatively lower amounts of TGF-β1 in the granuloma cells of egg-tolerized mice will also have implications for liver fibrosis due to the known fibrogenic properties of this cytokine (36). In murine schistosomiasis TGF-β1 has been shown to be elevated within the liver, and the cytokine is associated with fibrosis (37). Additionally, in TGF-β1 transgenic mice liver collagen synthesis is increased during schistosome infection (38). Collectively, our data suggest that the dominance of a type 1 cytokine phenotype concurrently associated with the presence of a diminished type 2 cytokine response impairs the development of schistosome egg granulomas and reduces collagen (fibrosis) formation in acutely infected mice.

We have recently shown that during the development of a type 2 cytokine-dominated response in schistosome infection there is a reduction in the relative frequency of IFN-γ-producing CD8+ T cells (Tc1 cells) and an expansion of IL-4-producing CD4+ T cells (Th2 cells) (8). In this study egg-tolerized mice had a type 1 cytokine profile by day 47 after infection, which is in marked contrast to the predominance of a type 2 cytokine response at this time of infection in normal mice (Fig. 4). The type 1 cytokine-dominated responses in egg-tolerized mice were in part due to a failure to reduce the number of Tc1 cells (Fig. 6). This inability to down-regulate these cells may be due to the absence of IL-10. Recently, it has been shown that during this acute stage of infection IL-10 may mediate activation-induced cell death of certain cell populations (9). The crucial role of IL-10 in murine schistosomiasis infection has been shown previously. Thus, treatment of schistosome-infected mice with exogenous IL-10 has been shown to directly regulate cellular responses and reduce immunopathological damage (39, 40). Additionally, Wynn and colleagues (26) have demonstrated that in the absence of IL-10 (IL-10-deficient mice) there is an elevated type 1 cytokine profile with impaired granuloma formation and increased pathology during acute schistosome infection. In double IL-10- and IL-4-deficient or IL-10- and IL-12-deficient animals the central regulatory role for IL-10 in responses to schistosome eggs has been further delineated (41).

In various infectious diseases it is becoming increasingly apparent that the intimate cytokine milieu regulates susceptibility and pathology during infection (42). In the context of schistosomiasis infection it is the egg granuloma that tightly regulates local cytokine production. Recently, it has been shown that IFN-γ production by cells within the granuloma is regulated by the presence of IL-4, IL-10, and TGF-β in the granuloma microenvironment (13). In this study granuloma cells from normal infected mice had a mixed production of type 1 and type 2 cytokines, with the coincident presence of IL-4 and IFN-γ-secretory T cells as well as cell-derived IL-10 and TGF-β1 (Table III). In contrast, cells from the liver granulomas of egg-tolerized mice secreted significantly more IFN-γ, TNF-α, and IL-12, with reduced IL-4, IL-10, and TGF-β1. Thus, the cytokine environments of the granulomas of egg-tolerized mice are type 1 cytokine dominated, which may be due to the absence of IL-4 and conversely the presence of elevated IL-12 within the granuloma. The increased secretion of IL-12 by granuloma cells from egg-tolerized mice is probably relevant to liver damage, as in other type 1 cytokine-induced models of severe liver injury a central role for IL-12 has been shown (43). The schistosome granuloma, via, for example, the known counter-regulatory role of IL-4 and IFN-γ on TGF-β (44), is a tightly controlled event that is dysfunctional in tolerized mice.

The tolerization method adopted in this study (Cy plus Ag) has previously been shown to influence B cell function and impair Ag-specific Ab responses (21, 22). Consistent with an effect on B cells, infected egg-tolerized mice had significantly reduced levels of Ab to egg Ag in ELISA. This humoral defect was Ag specific, as egg-tolerized mice had similar levels of total IgG to worm Ag as control animals. However, the anti-worm Ab response was type 1 isotype biased, with elevated IgG2a and diminished IgG1 and IgE in infected tolerized mice relative to those in control animals. As IgG2a isotype responses are stimulated by IFN-γ (45–47), the IFN-γ-dominated responses throughout schistosome infection of tolerized mice will have promoted the expression of this isotype. Conversely, the absence of type 2 cytokines (IL-4 and IL-13) that regulate class switching to IgG1 and IgE (48) in tolerized mice will have prevented the expansion of these isotypes during infection.

The major pathology observed in egg-tolerized mice was extensive damage to the liver. During experimental schistosome infections of nude, SCID, and T cell-depleted mice there is also extensive hepatotoxicity, which is characterized by similar cell damage and microvesicular steatosis as described here (2–5). Although it is not known whether similar liver pathology occurs in humans, individuals coinfected with schistosomiasis and HIV have raised plasma transaminase levels, suggesting elevated hepatocyte damage in immunocompromised humans (49). In all these cases of hepatotoxicity there is a defect in anti-egg immune responses during infection and a quantitative and qualitative difference in the granulomatous response to the egg. Egg-tolerized mice start to die within 2 wk of eggs arriving in the liver; thereafter, 50% of mice succumb to liver damage within 1 wk. The absence of Ab to egg Ag in tolerized mice is of particular significance with respect to the hepatotoxicity in these mice. T cell- and CD4+-depleted mice also have diminished Ab responses to egg Ag, and these mice suffer severe microvesicular damage during infection; this hepatocyte damage can be prevented by the transfer of serum from infected mice (18) (P. G. Fallon, unpublished observations). It is relevant that this hepatocyte damage occurs in immunologically intact mice once egg laying starts and eggs are deposited in the liver (~30–35 days after infection), but in normal mice the hepatocyte damage is transient and is resolved by emerging Ab and/or type 2 cytokine responses. Thus, the presence of anti-egg Ab during schistosome infection may have a crucial role, by neutralizing/removing secreted egg Ag, in ameliorating hepatocyte damage. A potential protective role for Ab has also been shown during schistosome infection of B cell-deficient (μMT) mice. These mice have enlarged granulomas and elevated hepatic collagen with increased mortality compared with wild-type mice. This pathology was not associated with a change in the type 1/type 2 cytokine balance (50). However, it is relevant that in a separate study in a different B cell-deficient mouse (JH locus deleted) there was a type 1 cytokine-dominated response during acute schistosome infection with normal granulomatous responses (51). Jankovic and colleagues suggest that as FcR-deficient mice also have similar liver pathology as μMT mice the role of Ab in regulating the granulomatous response to schistosome eggs is via Ab directly stimulating the production of anti-inflammatory mediators from FcR+ cells (50, 52).
As shown in this study egg-tolerized mice have a dramatically impaired (>90%) capacity to excrete parasite eggs. The schistosome egg excretion process requires eggs that are laid by adult worms in the mesenteric vasculature to transverse the intestinal wall to enter the gut lumen. Egg excretion is an immune-dependent process, with reduced excretion of eggs in T cell-deprived mice (16), SCID mice (5), more recently in B cell-deficient (μMT) mice (50), and CD4+ cell-deprived mice (P. G. Fallon et al., unpublished observations). Data from schistosome-infected humans support the hypothesis that egg excretion is immune dependent, with individuals with reduced CD4+ cells having reduced egg excretion (49). In egg-tolerized mice and CD4+ T cell-deprived animals there is a diminished granulomatous response associated with reduced type 2 cytokines and a quantitative defect in anti-egg Ab production during schistosome infection (this study and our unpublished observations). One mechanism by which type 2 cytokines could be active in facilitating schistosome egg excretion is via IL-5-mediated eosinophil production (53). In this study egg-tolerized mice have limited IL-5 secretion, and although we have not quantified blood eosinophils there were fewer eosinophils within the egg granuloma. The presence of eosinophils in the intestine has been implicated in the passage of eggs through the intestine (54). Thus, the absence of anti-egg immune responses in egg-tolerized mice is clearly a factor in the impaired egg excretion of these animals. However, it is also probable that the elevated gut collagen formation in these animals is relevant. Cy treatment kills dividing cells, and as the gut is a site of rapid cell turnover, there will be some intestinal cell damage and fibrosis in Cy-treated mice. In this study control mice that were thymectomized and Cy treated but not injected with eggs had the same elevated intestinal collagen levels as tolerized mice, but these animals had normal egg excretion. Additional studies are required to elucidate the immune mechanisms that control passage of schistosome eggs through the intestine.

In conclusion, this experimental study demonstrates that rendering mice unresponsive to eggs causes elevated type 1 cytokine responses with diminished type 2 responses during schistosome infection, and these effects are detrimental to the host. The results reported here support observations in a previous study in IL-4-deficient mice (55). This study has demonstrated that the normal stimulation of type 2 cytokines by eggs during schistosome infection is essential to regulate the proinflammatory type 1 cytokines that are elicited during early schistosome infection.

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