The Schistosome Granuloma: Characterization of Lymphocyte Migration, Activation, and Cytokine Production


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**References**

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The Schistosome Granuloma: Characterization of Lymphocyte Migration, Activation, and Cytokine Production


Granuloma formation and its regulation are dependent on lymphocytes. Therefore, we compared the characteristics of lymphocytes derived from the spleens and granulomas of Schistosoma mansoni-infected mice during the course of their disease. We examined lymphocyte cell cycle kinetics, migration, expression of activation Ags (CD69 and IL-2R), cytokine production (IL-2, IL-4, IFN-γ), and apoptosis. Lymphocytes in the G2/M phase of the cell cycle and high levels of lymphocyte intracellular IL-2 were found in the spleen but not in the granuloma. Cell trafficking experiments showed Ag-specific recruitment of schistosomal egg Ag (SEA)-reactive lymphoblasts into granulomas in vivo, as well as recruitment to, residence within, and egress from granulomas in vitro. Granuloma-derived lymphocytes were more highly activated than splenic lymphocytes based on higher levels of CD69 and IL-2R expression. While the granuloma microenvironment was rich in Th2 cytokines, during peak granuloma formation, the lymphocytes per se from the spleen and granuloma did not exhibit a dominant Th1 or Th2 cytokine profile, producing low but similar levels of IL-4 and IFN-γ. The discrepancy between high IL-2R expression and low levels of IL-2 protein production by granuloma lymphocytes was associated with increased apoptosis in the granuloma compared with the spleen. These findings support the hypothesis that granulomas may play a role in the regulation of systemic pathology in schistosomiasis by adversely affecting the survival of SEA-reactive, immunopathogenic T lymphocytes. The Journal of Immunology, 1998, 161: 4129–4137.

Materials and Methods

Experimental animals and infection with Schistosoma mansoni cercariae

Female C57BL/6 (Harlan Sprague-Dawley, Indianapolis, IN) were used in all experiments. Mice (5–6 wk of age) were infected with 30 cercariae percutaneously by tail immersion, according to the method of Bruce and Radke (15). Infected snails were obtained from Dr. Fred Lewis (Biomedical Research Institute, Rockville, MD).

Cell preparation

Mice were sacrificed at various times p.i., and their spleens and livers were aseptically removed. Spleens were pressed through 60 mesh stainless steel screens and the resultant cell suspension washed two times and suspended in complete medium (RPMI 1640 supplemented with 1 mM sodium pyruvate, 100 μg/ml streptomycin sulfate, 100 U/ml penicillin, 50 μg/ml gentamicin, 2 mM L-glutamine, 5 x 10^{-5} M 2-ME, 1% nonessential amino acids, and 10% heat-inactivated FCS). Livers were minced in a Waring blender at low speed for 15 s, and granulomas were collected by sedimentation. After extensive washing, granulomas were suspended in 10 ml complete medium and collagenase type IV (2.5 mg/ml; Sigma, St. Louis, MO), was added to both granulomas and splenic cell suspensions with subsequent incubation for 1 h at 37°C with frequent, gentle agitation. The cell...
suspensions were filtered through a 100-µm Nytex membrane (LabCor Products, Gaithersburg, MD) and washed in complete medium. Following RBC lysis with Tris-NH₄Cl, the cells were washed and viable cells were enumerated by trypan blue exclusion.

Cell migration studies

**In vivo studies:** Splenocytes from *S. mansoni* infected and keyhole limpet hemocyanin (KLH)-immunized mice. Donor lymphoblasts were prepared from bulk cultures of splenic suspensions using equilibrium density sedimentation (16). Lymphoblast separation was performed by resuspending 15 × 10^6 cells/ml in a solution of BSA (density, 1.082) that was overlaid with a less dense solution of BSA (density, 1.060). Gradients were spun to equilibrium at 10,000 × g for 30 min at 4°C. The less dense lymphoblasts were removed from the interface. B cells were depleted by a selection step with CD45R (B220) micro beads (Miltenyi Biotec, Auburn, CA). The purity and viability of the lymphoblasts was determined by size, granularity, phenotypic characteristics, surface Ig staining, trypan blue exclusion, and [³H]thymidine incorporation in response to specific Ag. Using these criteria, the transferred populations were consistently >90% viable lymphoblasts.

The lymphoblasts (2 × 10⁷) were labeled with the red fluorescent vital dye PKH-26 (2.2 × 10⁻⁶ M) (Zyn-R, Zynaxis Cell Science, Malvern, PA). The labeling reaction was terminated by the addition of FCS, and unbound dye was removed by density centrifugation. After washing, the cells were injected i.v. into the tails of recipient mice that had been exposed to 25 *S. mansoni* cercariae 10 wk before study or immunized with 100 µg of KLH in CFA wk before study. Four days before transfer, 10⁷ KLH-coated latex beads were injected into the portal vein of the KLH-sensitized mice. Eighteen hours after cell transfer, the animals were sacrificed and their lymph nodes, spleens, livers, and granulomas were obtained. Cell suspensions were prepared from each organ, and the number of labeled cells was determined by FACS.

**In vitro studies.** In vitro granulomas were studied using SEA-coated latex beads. Spleen cells from animals, previously exposed to *S. mansoni* for 12 wk or immunized with KLH, were added to SEA or KLH-ag-coated beads to form in vitro granulomas (18).

Small numbers (~10⁰) of cloned Th1 cells, reactive to either SEA or KLH (19), were labeled with the fluorescent, hydrophobic dye, 1',1'-dioctadecyl 3,3',3',3'-tetramethyldifluoroborocaine perchlorate (Dil; Molecular Probes, Eugene, OR) and were added to the culture (20, 21). Individual cells were identified by fluorescence and tracked at 4-s intervals using videomicroscopy. The identity of the labeled cell was confirmed by intermitting fluorescence microscopy. Images were obtained with a Dage-MTI (Michigan City, IN) model 65 videocamera, a JVC (Tokyo, Japan) Br-9000 VCR, Sony PVM-91 monitor (Tokyo, Japan), and Opti-quip (Highland Mills, NY) infra-red stage-mounted incubator. The migratory pathway was digitized, electronically calibrated, and measured with a KeE model 620005 compensating planimeter (Keuffel and Esser, Germany). An index of directed migration or persistence was calculated by dividing the net migration that was directed toward the central point of the bead by the total migratory path. An index of +1 meant that the cell migrated directly to the bead. An index of 0 meant that the migration was random. An index of −1 meant that the cell migrated directly away from the bead. The migration index was calculated within concentric rings of increasing diameter around the in vitro granuloma, i.e., 0–25 µm, 25–50 µm, 50–100 µm, 100–150 µm, etc.

**Flow cytometry**

In all flow cytometry determinations, individual isotype controls were included and photomultiplier tube voltage and gain settings initially adjusted such that isotype control values were near 10 fluorescence units. In all data reported here, lymphocyte gates were defined by forward and side light scatter characteristics, and background staining levels were between 5 and 15 fluorescence units.

**Cell cycle kinetics.** After fixation and permeabilization of cells (1 × 10⁶), cellular DNA was stained by incubating cells in staining buffer (PBS + 2% FCS) containing 10 µg/ml propidium iodide (Sigma) and 5 µg/ml RNase A (Sigma) for 30 min (22) before analysis by flow cytometry. Data were analyzed using ModFit version 2.0 (Verity Software). Forty percent of the splenic lymphocytes were in S phase and 16.5% were in G₂/M, whereas 47% of the granuloma lymphocytes were in S phase and 3.6% are in the G₂/M phase. Less than 1% of splenic lymphocytes obtained from uninfected animals were in S phase.

**Apoptosis.** Apoptosis was quantitated using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) methodology described by Goreszca et al. (23), with modifications. Cells were prepared, surface stained, and permeabilized as above. Cells were then incubated with 50 µl of TdT reaction mixture containing 0.1 M sodium cacodylate (pH 7.2), 2 mM CoCl₂, 0.2 mM DTT, 0.1 mg/ml BSA, 5 units TdT, and 0.3 nmol Cy5-dUTP (Amersham, Arlington Heights, IL) for 1 h at 37°C. After staining, the cells were washed three times with SB and analyzed by FACS. A control sample that contained all components of the reaction mixture except the TdT enzyme served as the negative control.

**Results**

**Lymphocyte cell cycle kinetics**

We first wished to ascertain the site of generation of the lymphocytes which were recovered from the spleens and granulomas of *S. mansoni*-infected mice. Cell cycle kinetics of gated lymphocyte populations from 8-wk-infected animals were analyzed. Spleen and granuloma cells were sorted by flow cytometry, stained with Biebrich scarlet and hematoxyline, and were 91–97% small lymphocytes by morphologic criteria. As shown in Figure 1, similar percentages of lymphocytes were in G₀/G₁ phase from the spleen.

**FIGURE 1.** Cell cycle kinetics of lymphocytes obtained from spleens and granulomas. The cell cycle kinetics of lymphocytes obtained from the spleen and granulomas of 8-wk-infected animals were determined using propidium iodide to determine cellular DNA content as described in Materials and Methods. Data were analyzed using ModFit version 2.0 (Verity Software). Forty percent of the splenic lymphocytes were in S phase and 16.5% were in G₂/M, whereas 47% of the granuloma lymphocytes were in S phase and 3.6% are in the G₂/M phase. Less than 1% of splenic lymphocytes obtained from uninfected animals were in S phase.
between the value shown by PKH-2
of splenic lymphocytes were in G2/M, whereas obtained from spleens and granulomas, were compared, over 16% maintained in culture with APCs, and assayed for viability and function. PKH-2 additional cells were not labeled, negatively selected by FACS, washed, and similarly assayed for function.

In contrast a large difference was observed in the numbers (44%) and granuloma (50%). Forty percent of splenic lymphocytes (Fig. 1A) and 46% of granuloma lymphocytes (Fig. 1B) were in S phase. In contrast a large difference was observed in the numbers of lymphocytes in the G2/M phase of the cell cycle. When cells, obtained from spleens and granulomas, were compared, over 16% of splenic lymphocytes were in G2/M, whereas <4% of granuloma lymphocytes were in G2/M (p < 0.01). These findings, coupled with the observation that there were many more lymphocytes per 10,000 total FACS events in spleens (3,857 ± 1,340) than in granulomas (403 ± 308) suggested that the vast majority of lymphocyte division was occurring in the spleen while few if any lymphocytes were dividing in the granulomas. Moreover, this division was contingent on infection by S. mansoni since >98% of spleen cells from naive animals were resting in the G0/G1 stage of the cell cycle (data not shown).

Cell trafficking

In vivo studies. Since our initial studies suggested that lymphocyte division was occurring in the spleen, we next sought direct evidence for specific migration of Ag-reactive lymphocytes from the spleen to the granulomas.

In vivo trafficking experiments were performed using SEA-reactive lymphocytes labeled with the red fluorescent vital dye PKH-26 (17) to determine whether these cells could be actively recruited to the schistosome granuloma. We first confirmed that the labeling and flow cytometry sorting procedures had no significant deleterious effects on the function of the transferred cells. To determine whether cell labeling altered cell function, an SEA-reactive Th1 clone, G4 (24), was labeled with PKH-26. Viability, proliferation in response to Con A or SEA stimulation, IL-2R expression, and in vitro granuloma formation around SEA-coated beads were examined (Table I). The viability of G4 cells was unchanged as a result of PKH labeling. Both labeled and unlabeled G4 cells responded to Con A or SEA stimulation with similar levels of proliferation. Similar IL-2R expression was observed in labeled and nonlabeled cells and the in vitro granuloma index was not affected by labeling with PKH.

After in vitro experiments had demonstrated that both labeling of cells with PKH and FACS sorting had no deleterious effects, in vivo trafficking experiments were performed. Donor lymphoblasts were prepared from S. mansoni or KLH-immunized mice and transferred i.v. to mice 10 wk after exposure to S. mansoni. After 18 h, the recipient mice were sacrificed. Cell suspensions were prepared and analyzed by FACS to determine the number of PKH-

<table>
<thead>
<tr>
<th>Cell Tested</th>
<th>Viability</th>
<th>ConA</th>
<th>SEA-AMBT</th>
<th>SEA-FACS</th>
<th>IL-2R</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKH-2</td>
<td>98 ± 3</td>
<td>29 ± 4</td>
<td>16 ± 2</td>
<td>3.5 ± 0.3</td>
<td>347 ± 26</td>
</tr>
<tr>
<td>PKH-2</td>
<td>97 ± 4</td>
<td>35 ± 5</td>
<td>19 ± 4</td>
<td>3.0 ± 0.4</td>
<td>291 ± 37</td>
</tr>
</tbody>
</table>

* Th1 clone G4 was labeled with PKH-2, positively selected by FACS, washed, maintained in culture with APCs, and assayed for viability and function. PKH-2 additional cells were not labeled, negatively selected by FACS, washed, and similarly assayed for function.

* Percent viable cells after 48 h of incubation with SEA and APCs, using criteria of phase-contrast microscopy and trypan blue exclusion.

* Response to Con A at 48 h: [3H]-thymidine incorporation: cpm × 106; mean ± 2 SEM.

* Ag-mediated proliferative response to SEA at 72 h: [3H]-thymidine incorporation: cpm × 106; mean ± 2 SEM.

* In vitro granuloma formation around SEA-coated beads: granuloma index ± 2 SEM (18).

* IL-2R expression: photometric Units ± 2 SEM. There is no significant difference between the value shown by PKH-2 and PKH-2 cells for any criteria of evaluation: p ≥ 0.05: Student’s t test.

FIGURE 2. SEA-reactive lymphoblasts specifically traffic to S. mansoni granulomas. Mice were exposed to S. mansoni or immunized against KLH and injected i.p. with 107 KLH-latex-coated beads to produce S. mansoni and KLH granulomas, respectively. The mice were then injected via their tail veins with 107 PKH26-labeled lymphoblasts with specificity for SEA (anti-SEA LB) or KLH (anti-KLH LB) or with lymphoblasts that had been heat killed (HK) (20 min at 56°C). After 18 h the mice were sacrificed and cell suspensions prepared from their combined axillary, inguinal, mediastinal, and periaortic lymph nodes, spleen, liver, or granulomas. The results are expressed as the percent of cells obtained from each organ ± SEM.

labeled cells in each organ (Fig. 2). When anti-SEA lymphoblasts were used as the source of transferred cells, >15% of the recovered labeled cells were found in the SEA granulomas compared with <3% in the KLH granulomas. When anti-KLH lymphoblasts were the source of transferred cells, ~12% of the recovered cells were located in the KLH granulomas and <3% were in the S. mansoni granulomas. Using heat-killed anti-SEA cells as the source of transferred cells resulted in the recovery of <5% of the cells in the S. mansoni or KLH granulomas and nearly 75% of the cells from the liver. Significant numbers of heat-killed cells were also seen in histologic sections of the lung; however, for technical reasons, these cells could not be recovered efficiently for FACS quantitation. The distribution of SEA-reactive lymphoblasts in normal, unsensitized animals were similar to those observed in KLH-sensitized mice and for KLH-reactive lymphoblasts in normal and S. mansoni-infected mice (data not shown). These studies clearly demonstrated the preferential accumulation of SEA-reactive lymphoblasts in S. mansoni granulomas due to active recruitment and retention within S. mansoni granulomas.

In vitro studies. If lymphocytes were generated systemically and migrated to the granulomas, wherein the granuloma might exert effects on these cells, then the granulomas could become potential regulatory organelles. In vivo studies to assess recirculation and residence time were not feasible since mice have recirculation times of less than 4 s. Therefore, in vitro studies were performed to see if cells could migrate specifically to the granuloma and subsequently leave these granulomas. Cloned T lymphocytes reactive to SEA or KLH (19) were labeled with Dil and followed with time-lapsed videomicroscopy to assess their directed migration toward, residence within, and egress from in vitro granulomas. An index of directed migration was determined for cells as they approached the in vitro granuloma. SEA-reactive T clones demonstrated dramatically increased directed migration as they neared the source of SEA; the SEA-coated bead (Fig. 3). The index of
granulomas.

Directed migration was measured in finite concentric rings around the granulomas. DiI-labeled cloned anti-SEA or anti-KLH lymphocytes were added to the cultures after 3 days and traced by videomicroscopy. An index of directed migration was calculated as cells left the granuloma. The initial ingress index for SEA-reactive cloned T cells from SEA granulomas was 0.62 ± 0.3 h, suggesting that egress of SEA-reactive T clones from SEA granulomas was slow, indicating that egress of SEA-reactive lymphocytes from SEA granulomas was a true directed migration and not simply the random movement of cells toward the SEA-coated bead.

Table II. *In vitro* ingress, residence, and egress patterns

<table>
<thead>
<tr>
<th></th>
<th>SEA Granulomas</th>
<th>KLH Granulomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag</td>
<td>Ingress</td>
<td>Residence</td>
</tr>
<tr>
<td>SEA</td>
<td>0.79 ± 0.09</td>
<td>38.4 ± 7.4</td>
</tr>
<tr>
<td>KLH</td>
<td>0.35 ± 0.13</td>
<td>6.2 ± 3.8</td>
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</table>

FIGURE 3. Cloned SEA-reactive T lymphocytes demonstrate increased directed ingress toward in vitro granulomas. C57BL/6 mice were exposed to 25 S. mansoni cercaria. Twelve weeks after exposure their spleen cells were obtained and used to produce in vitro granulomas around SEA-coated latex beads. Dil-labeled cloned anti-SEA or anti-KLH lymphocytes were added to the cultures after 3 days and traced by videomicroscopy. An index of directed migration was measured in finite concentric rings around the granulomas.

Directed migration approached 1.0; i.e., the SEA-reactive T clones moved directly toward the SEA-coated bead. Conversely, T clones, which were reactive to KLH, showed more random migratory patterns until they came very close to the SEA-coated bead. Reciprocal egress migration patterns were observed for SEA-reactive cells in KLH granulomas (Table II). Therefore, the SEA-reactive lymphocytes could be specifically sequestered and retained in SEA granulomas ($p < 0.01$).

The cells were next studied for their ability to leave the granulomas. An index of directed migration was calculated as cells left the granuloma. The initial egress index for SEA-reactive cloned T cells from SEA granulomas was slow, suggesting that egress of SEA-reactive T clones from SEA granulomas was initially retarded. Egress accelerated as the cell moved away from the granuloma (Fig. 4) and migration was random after the cell had moved over 300 μm from the SEA-coated bead. During the course of the egress studies, the granulomas were actually growing in size with the majority of cells moving toward the granulomas (18) (data not shown).

The data indicated that the migration and residence patterns of the SEA- and KLH-reactive clones were contingent upon the antigenic composition of the granulomas.

FIGURE 4. Cloned SEA-reactive T lymphocytes demonstrate directed egress from in vitro granulomas. C57BL/6 mice were exposed to 25 S. mansoni cercaria. Twelve weeks after exposure their spleen cells were obtained and used to produce in vitro granulomas around SEA-coated latex beads. Dil-labeled cloned anti-SEA or anti-KLH lymphocytes were added to the cultures after 3 days of culture and traced by videomicroscopy. Granulomas were observed until a Dil-labeled cell entered a granuloma. That cell was tracked by videomicroscopy. An index of directed migration was measured in finite concentric rings around the granulomas.

The labeled cells in the granulomas were next studied. Granulomas were produced in cultures containing 1000 unlabeled spleen cells per labeled, SEA-reactive, cloned T cell. In vitro granulomas were studied as soon as a single labeled cell had entered the granuloma to determine the mean residence time of the labeled cell within the in vitro granuloma (Table II). The mean residence time of the SEA-reactive Th1 cells within the SEA granuloma was 38.4 ± 7.4 h. The mean residence time of the anti-KLH cells which entered the SEA granuloma was 6.2 ± 3.8 h. Analogous reciprocal differences in residence times were observed for SEA-reactive cells in KLH granulomas and for KLH-reactive cells in KLH granulomas (Table II). Therefore, the SEA-reactive lymphocytes could be specifically sequestered and retained in SEA granulomas ($p < 0.01$).

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<th>Ingress</th>
<th>Residence</th>
<th>Egress</th>
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<tr>
<td>SEA</td>
<td>0.79 ± 0.09</td>
<td>38.4 ± 7.4</td>
<td>−0.73 ± 0.14</td>
</tr>
<tr>
<td>KLH</td>
<td>0.35 ± 0.13</td>
<td>6.2 ± 3.8</td>
<td>−0.28 ± 0.04</td>
</tr>
</tbody>
</table>

# Ag specificity of T clone.
# Ag specificity of in vitro granuloma: SEA-coated beads.
# Ag specificity of in vitro granuloma: KLH-coated beads.
# Index of directed ingress migration ± SEM measured 0–100 μm from the surface of the bead.
# Average residence time in granuloma: hours ± SEM.
# Index of directed egress migration ± SEM measured 0–100 μm from the surface of the bead.
granulomas of the state of activation of lymphocytes obtained from spleens and migration to and residence within granulomas. We first assessed changes occurred in these lymphocytes as a consequence of their experiments. We next wished to determine whether any functional

initial studies showed that cells could be obtained from spleens injected into animals and subsequently recovered in vivo from granulomas. These trafficking patterns were confirmed by in vitro experiments. We next wished to determine whether any functional changes occurred in these lymphocytes as a consequence of their migration to and residence within granulomas. We first assessed the state of activation of lymphocytes obtained from spleens and granulomas of S. mansoni-infected mice. We used three criteria of activation: expression of the early activation marker CD69 (25, 26); expression of a later, more functional activation marker, the IL-2R (CD25) (27, 28); and production of IL-2.

Expression of the early activation Ag CD69

Lymphocytes were assessed by multiparameter FACS for quantitative phenotypic evidence of activation. The kinetics of surface CD69 expression by spleen and granuloma lymphocytes obtained from animals 6 to 26 wk p.i. are shown in Figure 5. Data are expressed as the fold change in mean channel fluorescence (MCF) of CD69-stained samples over isotype-matched control samples. A MCF of 1 indicates no significant CD69 expression. At 7 wk p.i., granuloma lymphocytes expressed \( \sim 1.5 \times \) higher levels of CD69 than splenic lymphocytes. CD69 expression on granuloma lymphocytes increased in subsequent weeks p.i. and was maximal 10 wk p.i. when a 4.5-fold change in MCF was observed. Examination of CD69 expression on splenic lymphocytes showed a 2-fold increase in CD69 MCF very early in granuloma formation (6–8 wk p.i.). The relatively low level of CD69 expression observed on splenic lymphocytes from 7–9 wk p.i. corresponded with the higher levels of CD69 expression in granuloma lymphocytes. From 8 to 10 wk p.i., CD69 expression increased on splenic lymphocytes and reached a peak level of expression around 10.5 wk p.i., at the time of maximum granuloma formation. After 11 wk p.i., CD69 expression steadily declined on splenic lymphocytes whereas CD69 expression on granuloma lymphocytes remained relatively constant. Since 10,000 events were counted in each FACS determination, a 1% change in MCF is significant. These observations suggested that lymphocytes were more highly activated in the granuloma.

Expression of IL-2R

After examination of CD69 expression, we studied the expression of the IL-2R, a more specific marker of immune activation. Ag activation of T lymphocytes results in synthesis of the \( \alpha \)-chain and up-regulation of the \( \beta \)- and \( \gamma \)-chains to form the high affinity, heterotrimeric IL-2R complex (29, 30). We determined the percentage of splenic and granuloma lymphocytes which were positive for the IL-2R \( \alpha \)- or \( \beta \)-chain as a function of weeks p.i. A single group of animals was infected with S. mansoni and serially sacrificed. IL-2R \( \beta \)-chain (CD122) and IL-2R \( \alpha \)-chain (CD25) expression was examined on splenic and granuloma lymphocytes (Fig. 6, A and B). The data shown in Figure 6 are representative of at least three separate groups of animals that were infected and serially examined at various weeks p.i. The percentage of IL-2R \( \beta \)-chain-positive lymphocytes in the spleen remained relatively constant, around 2%, during the course of infection. A much different result was observed in the granuloma. At 6.5 wk p.i., \( \sim 4.5\% \) of the granuloma lymphocytes were IL-2R \( \beta \)-chain positive. This percentage increased dramatically with increasing weeks p.i., reaching 15% at 9 to 10 wk p.i. After 10 wk p.i., the percentage of granuloma lymphocytes bearing the IL-2R \( \beta \)-chain began to decline and returned to \( \sim 4\% \). The patterns of IL-2R \( \beta \)-chain expression paralleled the kinetics of granuloma formation, which also peaked at 10 wk p.i. (31).

Expression of CD25 (IL-2R \( \alpha \)-chain) was also examined at various weeks p.i. CD25 expression on splenic lymphocytes peaked around 7 wk p.i., at which point \( \sim 25\% \) of the lymphocytes were positive for CD25 (Fig. 6B). After 8 wk p.i., CD25 expression on splenic lymphocytes decreased and remained relatively low during the remainder of the infection period studied. In the granuloma at 7 wk p.i., nearly 50% of the lymphocytes were positive for CD25, and maximal CD25 expression occurred around 9 wk p.i. After 9 wk p.i., the percentage of CD25\(^\text{+}\) lymphocytes decreased slightly.

**FIGURE 5.** Granuloma lymphocytes express higher levels of CD69 during the course of S. mansoni infection. Expression of the very early activation marker CD69 on lymphocytes from the spleen (○) or granuloma (■) at various weeks p.i. was measured by FACS. The fold change in MCF intensity was determined by dividing the MCF of CD69-stained cells by the MCF of an isotype-matched control tube. The data are expressed as the fold change in that ratio. Each data point represents the mean ± SEM of at least three observations.

**FIGURE 6.** Granuloma lymphocytes express higher levels of IL-2R during S. mansoni infection. Expression of the IL-2R \( \beta \)-chain (CD122) (A) and IL-2R \( \alpha \)-chain (CD25) (B) on lymphocytes from the spleen (○) and granuloma (■) at various weeks p.i. was measured by FACS. Data are expressed as the percentage of lymphocytes expressing the IL-2R \( \beta \)-chain or IL-2R \( \alpha \)-chain (CD25). Collectively, these two chains constitute the high affinity IL-2 receptor.
but remained >40%, which was ~3–4 times the percentages observed in the spleen at the same time. Table III compares the percentages of CD25 + lymphocytes from the spleen and granuloma at 7 to 8 wk p.i. vs 10 to 11 wk p.i. At both time points, significantly greater percentages of granuloma lymphocytes were CD25 + than were splenic lymphocytes. In addition, Table III shows that while the number of CD25 + lymphocytes remains relatively unchanged in the granuloma from 7 to 8 wk vs 10 to 11 wk, in the spleen approximately twice as many lymphocytes were CD25 + at 7 to 8 wk p.i. vs 10 to 11 wk p.i., suggesting that lymphocytes may be initially activated in the spleen from 7–8 wk p.i. and then are recruited to the granuloma. Since both the IL-2R β- and α-chains were expressed by granuloma lymphocytes, these cells expressed the high affinity IL-2R that characterizes activated Ag-reactive lymphocytes.

Cytokine production by lymphocytes
Our previous study using enzyme-linked immunospot (ELISPOT) analysis showed that high levels of cytokines were produced in the granuloma (32). Numerous other studies using ELISA assays and in situ hybridization have ascribed similar cytokine production primarily to Th2 lymphocytes and have suggested that cytokine production has significant effects on both local and systemic immunity (13, 33, 34). Therefore, we wished to determine the exact cytokine production profiles of splenic and granuloma-derived lymphocytes in S. mansoni-infected mice and to assess if the lymphocytes per se expressed a Th1 to Th2 profile, as defined by IFN-γ or IL-4 production. We used FACS to measure intracellular cytokine production by cells within the lymphocyte gate from acutely infected (10–12 wk p.i.) mice. Figure 7 shows typical cytokine staining patterns from 10 wk p.i. We measured intracellular IL-2, IL-4, and IFN-γ in freshly isolated splenic and granuloma lymphocytes without further in vitro stimulation. The results showed relatively high levels of IL-2 in the spleen, with significantly lower levels of IL-2 in the granuloma. Low but similar levels of IFN-γ were produced by both splenic and granuloma lymphocytes. IL-4 production was minimally elevated in granuloma lymphocytes compared with splenic lymphocytes. However, when data were pooled from multiple experiments (n ≤ 5) examining lymphocytes from 10 to 12 wk p.i., the only statistically significant difference in cytokine production observed was greater IL-2 production by splenic lymphocytes compared with granuloma lymphocytes. No statistically significant differences were measured for IL-4 or IFN-γ. Therefore, we observed no detectable Th1 or Th2 cytokine production preference by the unstimulated splenic or granuloma lymphocytes.

Apoptosis of lymphocytes
If the granuloma is an immunoregulatory organelle, it may act through the elimination of Ag-reactive lymphocytes. IL-2 withdrawal with resultant IL-2 desaturation is known to induce apoptosis. We had observed high levels of IL-2R expression along with low levels of IL-2 production in the granuloma lymphocytes. Therefore, we examined the survival of lymphocytes in granulomas by measuring lymphocyte apoptosis. Table IV shows the percentage of apoptotic splenic and granuloma lymphocytes, the expression of IL-2R, and the production of IL-2, measured 10 to 12 wk after exposure. In the spleen, where IL-2 production was high and IL-2R expression was relatively low, ~6% of the lymphocytes were apoptotic (n > 5). In the granuloma, where IL-2R expression was high and IL-2 protein was low, creating a microenvironment relatively deficient in IL-2, ~50% of the lymphocytes were apoptotic. These data suggested that lymphocytes in the granuloma

<table>
<thead>
<tr>
<th>Table III</th>
<th>Percentages of CD25 + lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks p.i.</td>
<td>Spleen*</td>
</tr>
<tr>
<td>7–8</td>
<td>21.54 ± 2.81e</td>
</tr>
<tr>
<td>10–11</td>
<td>12.02 ± 1.14e</td>
</tr>
<tr>
<td>p valuee</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* Cells obtained from spleen or granuloma.
+ Significance of difference between spleen and granuloma. Statistics were calculated by paired Student’s t test.
$ Data compiled from five separate experiments; mean ± SEM.
$ Significance of difference between wk 7–8 and wk 10–11 spleen or granuloma cells. Statistics were calculated by paired Student’s t test.

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Comparison of IL-2, IL-2R, and apoptosis in splenic and granuloma lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of Cells*</td>
<td>IL-2 MCFb</td>
</tr>
<tr>
<td>Spleen</td>
<td>3.25 ± 0.38</td>
</tr>
<tr>
<td>Granuloma</td>
<td>1.83 ± 0.28</td>
</tr>
</tbody>
</table>

* Cells were obtained from infected C57BL/6 mice 10–12 wk p.i. and prepared as described in Materials and Methods.
+ The fold change in MCF was determined by dividing the mean fluorescence of gated lymphocytes stained for intracellular IL-2 by MCF of gated lymphocytes stained with an isotype control Ab.
$ Apoptosis was quantitated by FACS using TUNEL methodology with Cy5-dUTP plus TdT enzyme. A negative control without TdT enzyme but with Cy5-dUTP was used.

$ Statistical accuracy, ±1%.
were more likely to undergo apoptosis than were lymphocytes in the spleen.

Discussion
The role(s) of granulomas in schistosomiasis have been the focus of intense study. Many functions have been ascribed to this lesion. On one hand, granulomas may play a protective role by sequestering hepatotoxins secreted from eggs (35, 36). On the other hand, the granulomatous response is a cell-mediated inflammatory response that may result in tissue fibrosis and pathology (37–40). Indeed, our earlier studies in athymic mice (41) have shown that immunopathology is actually less in athymic mice than in heterozygotes and thymic-reconstituted mice. Additional studies have shown that the granuloma may be essential for conveying viable eggs through tissues to complete the parasite life cycle (42). In this study, we propose an additional potential role of the granuloma to be function as an immunoregulatory antipathology organelle. As an immunoregulatory organelle, the granuloma may function to both activate and/or destroy Ag-reactive cells during the course of S. mansoni infection. Thus the granuloma may be a site of activation and immunopathogenesis or a site for inactivation of potentially immunopathogenic cells. In the latter context one of the primary immunoregulatory roles of the granuloma would be to sequester and/or eliminate Ag-reactive lymphocytes in an attempt to protect host tissues from the pathologic consequences of over-production of inflammatory cytokines by Ag-reactive lymphocytes. Our findings support such a hypothesis.

Our initial studies were aimed at defining the site of generation of SEA-reactive lymphocytes. While it is true that granulomas form around eggs located in the liver, schistosome Ag and circulating immune complexes exist in the bloodstream and Ag-reactive lymphocytes can be found in the spleen (43–45). Therefore, it is possible that Ag-driven cell division could occur at any site. When spleen cells from naive animals were examined, >98% of the lymphocytes were resting in the G0/G1 stage of the cell cycle (data not shown). Our cell cycle kinetics data obtained from S. mansoni-infected animals showed that ~40% of splenic and granuloma lymphocytes were in S phase but only splenic lymphocytes were capable of reaching the G2/M phase of the cell cycle. These results implied that lymphocytes were undergoing division in the spleen but not in the granuloma. The absence of lymphocytes in the G2/M phase of the cell cycle in the granuloma suggested that the granuloma microenvironment was not conducive to successful cell division. Cells which were in S phase in the granulomas might either leave the granuloma to complete division elsewhere or may follow a pathway of development which preferentially leads to their elimination, perhaps through Ag-driven cell death (apoptosis).

If the granuloma is a regulatory organelle, it might influence pathology by locally sequestering and destroying pathogenic cells or by promoting the development of tolerogenic cells, which would leave the granuloma and act peripherally. Therefore, we examined the recruitment and migration of lymphocytes to the granuloma by means of in vivo and in vitro trafficking studies. Our trafficking data showed the preferential recruitment of SEA-reactive splenic lymphocytes to the granulomas in an antigenically specific manner. Since the circulation time in a mouse is less than 4 s, we were unable to assess directly the ability of activated lymphocytes to reenter the periphery or to undergo multiple recirculation cycles in vivo. However, our in vitro studies confirmed that cloned SEA-reactive lymphocytes migrated into granulomas, were specifically retained within granulomas, and subsequently migrated out of SEA granulomas.

Based on the expression of the activation Ags CD69 and the IL-2R, and the production of IL-2, our studies showed that lymphocytes were more highly activated in the granuloma than in the spleen. This preferential activation might be due to selective migration of the most highly activated cells from the spleen to the granulomas or due to augmented activation in the granuloma per se. Since cells demonstrating high levels of CD69 expression were never found in the spleen, the latter explanation is favored. These data further suggested that lymphocyte activation was being regulated in the granuloma. The increased levels of lymphocyte activation observed in the granuloma along with the presence of granuloma lymphocytes in the S phase but not in G2/M suggested that granuloma lymphocytes may be more susceptible to apoptosis, since prior activation and cell cycle entry are known criteria for apoptosis (46–48). In this context, the higher local Ag concentration and greater resultant T cell activation in the granulomas may be the stimulus for this apoptosis.

The host immune response to S. mansoni infection has been shown to be a T cell-dependent process (1, 2, 24, 49). Classically, the initial response involves a Th1-type response which has been shown to be directed against early stages of the parasite and to be important for the induction of cell-mediated protective immunity to S. mansoni (50–52). As the disease progresses, schistosomal SEA become recognized by specific granuloma forming CD4+ T lymphocytes (53, 54). Disease progression is also characterized by an increasing dominance of Th2 cytokines (IL-4, IL-5, granulocyte-macrophage CSF, IL-10) relative to Th1 (IFN-γ)-associated cytokines. This transition has been attributed to a change in Th1 to Th2 lymphocyte dominance (12, 13, 55, 56). The balance of Th1 to Th2 cytokines is important in the regulation of pathology, in particular granuloma formation and hepatic fibrosis (12, 13, 49, 55, 57–61), and may have systemic consequences as well (33, 34, 62, 63).

Other recent studies which have suggested non-CD4+ sources of IL-4 (64, 65) underscore the importance of precisely defining the cellular source(s) of cytokines in the particular experimental model being examined. In the current study we assessed lymphocyte cytokine production on a single cell basis using FACS to precisely define the cellular source(s) of the reported Th1 and Th2 cytokines at the level of 99% accuracy. We examined spleen and granuloma lymphocytes from acutely infected animals, immediately upon recovery, without further in vitro stimulation in an attempt to ascertain the true in vivo cytokine profiles as closely as possible. Our findings showed that the lymphocytes per se were not responsible for the reported Th2 cytokine dominance found in granulomas in S. mansoni-infected mice. However, we did observe a dominant Th2 cytokine profile in a nonlymphocyte population, and the results of those studies are the subject of a different report (32). The studies that have reported Th1 to Th2 cytokine dominance in lymphocytes employed artificial immunization models and/or in vitro stimulation in their cytokine assays. The significance of the potential for Th2 cytokine production after in vitro (12) stimulation and the strength of the analogy between findings based on the lung and liver granuloma models (66, 67) and in vivo events occurring as a consequence of infection, are problematic.

In our determinations, the profiles of cytokine production by splenic and granuloma lymphocytes were very similar with the exception of IL-2 production. Granuloma lymphocytes appeared to be relatively deficient in IL-2 production when compared with spleen-derived lymphocytes. IL-2 withdrawal with resultant IL-2R desaturation is known to be one mechanism whereby apoptosis is induced. In addition to IL-2 withdrawal, cellular activation and entry into the cell cycle are prerequisites for apoptosis (68–70). Since we initially observed a relative deficiency of IL-2 in the
granuloma accompanied by higher levels of IL-2R expression and activation, as well as the lack of successful lymphocyte division in the granuloma, we subsequently examined lymphocyte apoptosis as a possible mechanism whereby the granuloma might regulate cell survival and subsequent immunopathology. Our initial studies clearly showed that a higher percentage of granuloma lymphocytes were apoptotic than splenic lymphocytes. We are currently investigating the possibility that cytokine production may effect subsequent apoptosis by specific subpopulations of T cells.

In summary, these data suggest that the granuloma recruits and activates lymphocytes. Moreover, the granuloma may function as an immunoregulatory organule that regulates Ag-specific lymphocyte activation, cytokine production, and apoptosis. Splenic and granuloma lymphocytes did not exhibit a dominant Th2 cytokine production profile in these experiments, which examined cells without in vitro stimulation. We did observe a nonlymphocyte population that produced large amounts of Th2 cytokines in the granuloma, and these data will be the subject of a future report. Granuloma lymphocytes were more likely to undergo apoptosis than splenic lymphocytes, and we are investigating this phenomenon in greater detail to determine whether the granuloma might eliminate resident, potentially pathogenic, lymphocytes by IL-2-dependent, Fas/Fas ligand-mediated apoptotic mechanisms. The precise mechanism(s) of the induction of apoptosis in SEA-reactive lymphocytes sequestered in the granulomas and the systemic implications of that apoptosis are the subjects of ongoing studies.

References


