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Anti-L3T4 Antibody Treatment Suppresses Hepatic Granuloma Formation and Abrogates Antigen-Induced Interleukin-2 Production in *Schistosoma mansoni* Infection

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In murine schistosomiasis mansoni, granulomatous inflammation is an immune response that involves egg antigen presentation to T cells in the context of class II major histocompatibility complex determinants and subsequent inflammatory lymphokine production by delayed-hypersensitivity (T_{DH}) lymphocytes. In the present study, monoclonal antibodies directed against L3T4, I-A, and Lyt-2 molecules were injected intraperitoneally into *S. mansoni*-infected mice to study the role of these membrane antigens in the process of granuloma formation. A dramatic suppression of the hepatic granuloma size and antigen-induced interleukin-2 (IL-2) production by spleen cells was seen in mice that received anti-L3T4 monoclonal antibody treatment. The total number of cells, especially the $L3T4^+$ T cells, was greatly diminished in the spleens. Furthermore, histopathological study of the granulomas in stained liver sections demonstrated the paucity of eosinophils and macrophages, absence of epithelioid cells and multinucleated giant cells, and minimal collagen deposition within the lesions. Damaged hepatocytes were also seen surrounding these ill-formed granulomas. In contrast, anti-I-A monoclonal antibody treatment partially suppressed IL-2 production, although granuloma size and cellular composition remained the same. Mice that received anti-Lyt-2 monoclonal antibody did not show any changes in either IL-2 production or hepatic granulomatous inflammation. The data presented in this paper indicate a crucial role for L3T4 molecules present on a subset of class II major histocompatibility complex-restricted T_{DH} cells in IL-2 production and the generation of the granulomatous response.

Schistosomiasis mansoni is a major tropical helminthic disease characterized by the development of granulomatous inflammations around parasite eggs trapped in the liver and intestines (2, 20). The importance of delayed-hypersensitivity T (T_{DH}) cells in the process of granuloma formation has been experimentally demonstrated by thymectomy (9) and thymectomy and anti-lymphocyte (4) or anti-thymocyte (10) serum treatment, as well as by the use of nude mice (1, 5, 21). These studies demonstrated that T-cell deprivation or absence significantly suppressed the granulomatous inflammation. Analysis of T-cell subsets showed that T_{DH} lymphocytes, responsible for the generation of inflammatory lymphokine production and granuloma formation, belong to the $Lyt\text{-}1^+2^-$ subpopulation (6, 26).

The recently developed monoclonal anti-L3T4 antibody recognizes an epitope, L3T4a, which is found exclusively on a subset of T cells that respond to antigens in the context of class II major histocompatibility complex (MHC) gene products (7, 8). The effect of this monoclonal antibody (MA) was tested in two autoimmune disease models in mice. It was shown that repeated administration of anti-L3T4 MA either prevented or reversed the development of experimental allergic encephalomyelitis (EAE) or autoimmune nephritis (24, 28). Ongoing experiments in our laboratory indicate that a long-term, soluble egg antigen (SEA)-specific T_{DH} cell line, established from liver granulomas displays L3T4 molecules (S. Ragheb, R. C. Mathew, and D. L. Boros, Fed. Proc. **44**:1695, 1985). Therefore, we studied the significance of L3T4 molecules and I-A antigens on the immune interactions that lead to granulomatous inflammation.

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We report here that anti-L3T4 MA treatment given for 2 weeks during the early stages of *Schistosoma mansoni* infection dramatically suppressed antigen-induced interleukin-2 (IL-2) production and hepatic granulomatous inflammation. Anti-I-A MA treatment, on the other hand, did not affect the size of the granulomas, whereas it suppressed antigen-induced IL-2 production. These data indicate the importance of L3T4 molecules and I-A antigens in T_{DH} cell activation, lymphokine production, and generation of the granulomatous response.

MATERIALS AND METHODS

Mice. Female CBA/J mice (Jackson Laboratories, Bar Harbor, Maine) were used in all experiments. Food and water were provided ad libitum.

***S. mansoni* infection.** Eight-week-old mice were injected subcutaneously with 25 cercariae of the Puerto Rican strain of *S. mansoni*. The infected mice were given acidified drinking water.

MAs used for treatment. The following hybridomas were obtained from the American Type Culture Collection, Rockville, Md.: clone GK1.5 (TIB 207), secreting anti-L3T4 antibody (rat immunoglobulin G2b [IgG2b]); clone 10-2.16 (TIB 93), secreting anti-$I\text{-}A^k$ antibody (mouse IgG2b); and clone 39/3C2.2 (TIB 105), producing anti-Lyt-2 antibody (rat IgG2a). These hybridomas were grown in serum-free medium HB101 (New England Nuclear Corp., Boston, Mass.), and the immunoglobulins were partially purified from the supernatants (1 to 1.5 liters per hybridoma) by adding solid $(NH_4)_2SO_4$ with gentle stirring to 50% saturation for 0.5 h. The precipitates were spun down, dissolved in distilled water, dialyzed against Tris-NaCl buffer (0.05 M Tris, 0.15 M NaCl), pH 8.6, and the protein content was determined by measuring optical density at 280 nm.

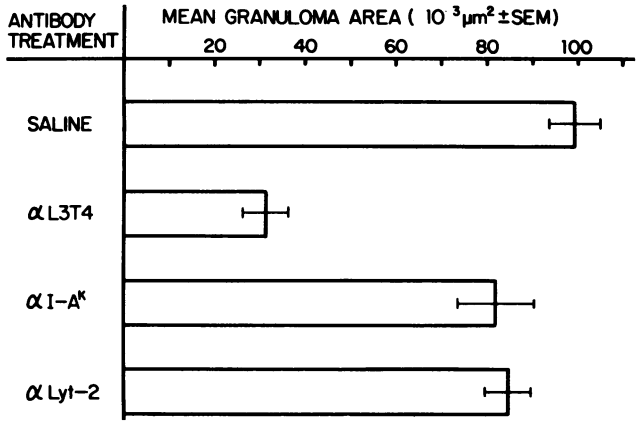

FIG. 1. Hepatic granuloma size in *S. mansoni*-infected mice treated with i.p. injections of saline, anti-L3T4 MA, anti-I-A^k MA, or anti-Lyt-2 MA. The injections were given on alternate days for 2 weeks between 6 and 8 weeks after infection. Bars represent the mean ± standard error of the mean (SEM) for 6 to 10 mice per group.

Antibody treatment. Six-week-infected mice were divided into three experimental groups of 10 mice each. Each group received anti-L3T4 MA, anti-I-A^k MA, or anti Lyt-2 MA intraperitoneally (i.p.) (500 µg of MA protein per 0.5 ml) on alternate days for 2 weeks. A control group of seven mice was given 0.5 ml of saline i.p. on the same day. Thus, each mouse received a total of seven injections. One day after the last treatment, the animals were sacrificed and their livers and spleens were used in the experiments.

Spleen cell counts and T-cell phenotypes. Total live cells in the spleens were determined by the eosin Y exclusion method. T cells were enumerated by a direct immunofluorescence technique with a mouse MA anti-Thy-1.2 conjugated to fluorescein isothiocyanate (FITC) (Miles Scientific, Naperville, Ill.). Rat hybridomas secreting anti-L3T4 MA and anti-Lyt-2 MA were grown in RPMI medium with 10% fetal calf serum. The supernatants were used in an indirect immunofluorescence assay with affinity-purified goat F(ab')$_2$ anti-rat IgG-FITC conjugate (Cooper Biomedical, Malvern, Pa.) to identify L3T4$^+$ and Lyt-2$^+$ cells.

Histology. Liver specimens were fixed in Bouin solution and processed for histology. Sections were made at multiple levels and stained with hematoxylin and eosin or Mallory trichrome stain. Histopathological findings were kindly evaluated and reported by Franz von Lichtenberg, Department of Pathology, Harvard University Medical School, from coded slides. The identity of slides was revealed only after the evaluation.

Granuloma measurement. Liver sections (5 µm thick) made at multiple levels were stained with hematoxylin and eosin. Granuloma area was calculated from the granuloma diameter measured with an automated image-splitting device (Vickers, Woburn, Mass.).

Granuloma harvest and preparation of granuloma cell suspension. Four livers from each group were homogenized in a Waring blender, and the released granulomas were treated with collagenase and dispersed into single-cell suspensions as described in detail previously (12). The cells were cultured overnight at 5 × 10^6 cells per ml in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.) so that cell membrane antigens that may have been altered by the collagenase treatment could be reexpressed.

IL-2 production. Four spleens per group were taken. Single-cell suspensions were made separately from each spleen, and cells were cultured at 5 × 10^6 per ml in 25-cm^2 tissue culture flasks with 5 µg of SEA per ml. The supernatants were collected 24 h later and stored at −70°C.

IL-2 assay. IL-2 content of the supernatant was determined by its ability to support the proliferation of IL-2-dependent CTLL20 cells by a modified method of Gillis et al. (13). CTLL20 cells (10^4 per well) in 100 µl of medium plus 100 µl of six dilutions of culture supernatants were apportioned into flat-bottom microtiter plates (Corning Glass Works) in quadruplicate. [^{3}H]thymidine ([^{3}H]TdR, 1 µCi per well; ICN Radiochemicals, Irvine, Calif.) was added after 20 h, and the cells were harvested 4 h later. Dilution curves were plotted for the samples as a percentage of maximum incorporation by a standard IL-2 preparation, and the units of IL-2 per milliliter were calculated by probit analysis (13). The standard IL-2 was prepared by stimulating Lewis rat spleen cells with concanavalin A (ConA) (5 µg/ml) for 24 h. The supernatant was batch absorbed with Sephadex G-10 to remove ConA and diluted 1:10 before use.

Lymphocyte proliferation assay. Spleen cells were cultured in round-bottom microtiter plates (Nunclon; Thomas Scientific, Philadelphia, Pa.) at 2 × 10^5 cells per well in 50 µl of medium with 2 µg of SEA or ConA in 20 µl. After 4 days for SEA and 1 day for ConA, 1 µCi of [^{3}H]TdR per cell was added, and 24 h later the cells were harvested onto glass filter papers for scintillation counting.

Statistical analysis. The Student *t* test was used to calculate significant differences between control and experimental groups. Values of $P \geq 0.05$ were considered not significant.

RESULTS

Suppression of granulomatous inflammation by anti-L3T4 MA treatment. To study the effect of MAs of different specificities on granuloma formation, 6-week-infected CBA/J mice were given 0.5-ml i.p. injections of antibodies directed to the L3T4, I-A, and Lyt-2 antigens on alternate days for 2 weeks. Compared with saline treatment, anti-L3T4 MA-treated animals demonstrated a dramatic 68% suppression ($P < 0.001$) of the granulomatous response around the eggs in the liver (Fig. 1). A similar regimen of anti-I-A MA or anti-Lyt-2 MA treatment in contrast did not cause any significant suppression of the granuloma size.

Histopathology of liver tissue from treated and control animals. The livers from saline-treated animals showed numerous large florid *S. mansoni* egg-induced granulomas containing lymphocytes, macrophages (including epithelioid and multinucleated giant cells), eosinophils, fibroblasts, and neutrophils, typical of early infection in an immunocompetent host (Fig. 2A). Mallory trichrome staining showed the presence of concentric bands of collagen within the granulomas (5). The hepatocytes surrounding the granulomas were normal except for occasional compression atrophy and mild autolytic artifacts. A few foci of infarctlike liver cell necrosis of the coagulative type were found, mostly in subcapsular regions and usually in association with triads containing many coalescing granulomas.

In contrast, livers from anti-L3T4 MA-treated animals showed profound changes in the host reaction to the entrapped eggs (Fig. 2B). There was little or no reaction around some eggs, although they were embryonated; others were surrounded by relatively small granulomas of dense appearance, containing mainly mixed mononuclear cells, i.e., lymphocytes, small macrophages with a high nucleus-to-cytoplasm ratio, and rare granulocytes of all classes. Signif-

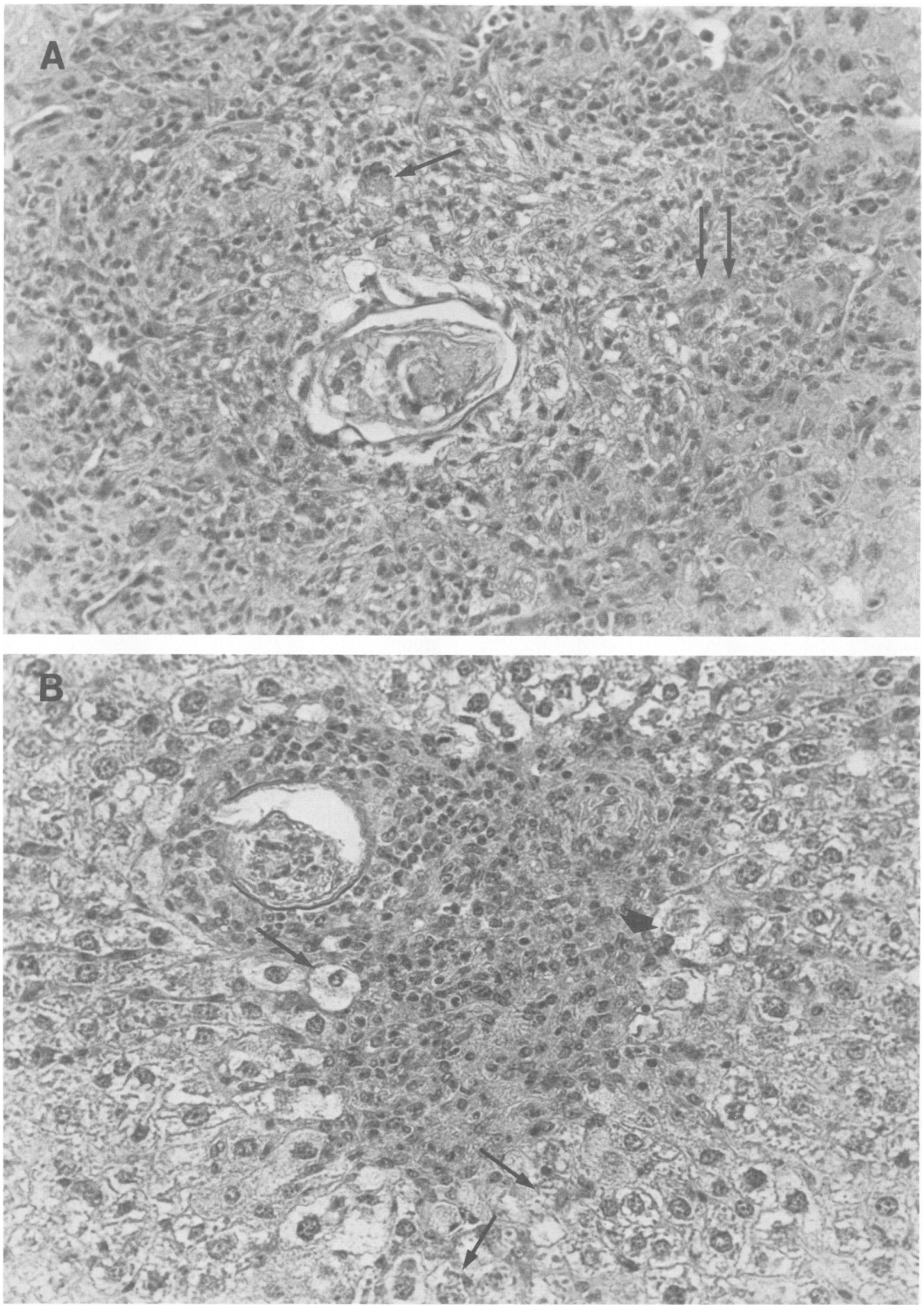

FIG. 2. *S. mansoni* egg-induced granulomas in the liver. (A) Saline-injected group. A typical granuloma in this group shows granulocytes, macrophages, lymphocytes, and fibroblast components, with a thin rim of relatively intact liver cells. Note the epithelioid macrophages (double arrows) and the multinucleated giant cell towards 12 o'clock from the egg (arrow). (B) Anti-L3T4 MA-treated group. Large, activated macrophages are absent, most of the macrophages are immature (arrowhead), and the granulocytic cells are scarce, making for a compact appearance. The hepatocytes at the granuloma rim show pycnotic nuclei (arrow) and microvesicular swelling. A Councilman body is seen at 6 o'clock (arrows). Fibroblastic activity was minimal. Both panels stained with hematoxylin and eosin (×540).

TABLE 1. T-cell phenotypes in the spleens of mice at 8 weeks postinfection

Treatment	Mean no. (10^6 of total spleen cells ± SE (n = 4)	Mean % of total cells ± SE		
		Thy-1.2^{+a}	L3T4^{+b}	Lyt-2^{+b}
Saline (control)	208.8 ± 78.7	22.2 ± 4.7	19.2 ± 3.2	8.7 ± 0.51
Anti-L3T4 MA	93.8 ± 18.9	17.7 ± 3.71	9.7 ± 1.79	13.7 ± 1.53
Anti-I-A^k MA	105.0 ± 28.9	20.1 ± 2.0	14.5 ± 0.78	9.3 ± 0.56
Anti-Lyt-2 MA	174.8 ± 39.4	20.9 ± 1.42	16.1 ± 4.15	4.0 ± 0.24

[a] Direct immunofluorescence with a monoclonal anti-Thy-1.2-FITC conjugate.
[b] Indirect immunofluorescence with specific rat MAs and goat F(ab')$_2$ anti-rat IgG-FITC conjugate (conjugate was adsorbed with mouse splenocytes to remove cross-reacting antibodies).

icantly, epithelioid cells and polykarions of macrophage origin were absent. Eosinophils were present but relatively sparse, as were fibroblasts. Mallory trichrome staining did not reveal bundles of collagen within these granulomas. Some of the granulomas were bordered by hepatocytes showing microvesicular change, with bloating and rounding of the cytoplasm. In addition, scattered acidophilic bodies (Councilman bodies) with pycnotic nuclei (or none) were found in the same granuloma border zone, representing individual liver cell necrosis. However, the bulk of the liver cells remote from granulomas appeared normal. Portal inflammatory infiltration was also much lower than in the control group, and plasma cells were either very sparse or absent.

Histologically, livers of anti Lyt-2 MA- and anti-I-A MA-treated animals were similar to those of saline-treated control animals.

Effect of anti-L3T4, anti-I-A, and anti-Lyt-2 MA treatment on the T-cell phenotypes of spleen and granuloma cells. To investigate whether the granuloma suppression was caused by the depletion of T cells, the number of T cells in the spleens of antibody-treated mice was determined. Compared with saline-injected mice, the proportion of both Thy-1.2$^+$ and L3T4$^+$ cells was lower in the spleens of anti-L3T4-treated animals, although the L3T4$^+$ decrease was more pronounced (Table 1). Since there was also a 54% decrease in the total number of spleen cells, a fourfold absolute reduction of L3T4$^+$ cells was observed in the spleens compared with the saline-treated animals. On the other hand, the proportion of Lyt-2$^+$ cells increased in the anti-L3T4-treated group, whereas it decreased in anti-Lyt-2-treated animals. Anti-I-A treatment also produced a 49% decrease in the total number of L3T4$^+$ cells in the spleens. In contrast, anti-Lyt-2 treatment caused a 4.7% decrease in the Lyt-2$^+$ cells but did not affect the other cell types.

Phenotypic characterization of the granuloma T lymphocytes was also attempted. The granulomas were dispersed with collagenase and cultured overnight to allow time for reexpression of any markers lost by collagenase treatment. The proportion of Thy-1.2$^+$ cells in the granulomas of the treated animals were as follows: saline, 9.6%; anti-L3T4, 22.7%; anti-I-A, 10.6%; and anti-Lyt-2, 19.2%. Whereas the count of Thy 1.2$^+$ T cells was consonant with previous observations, quantitation of the L3T4$^+$ and Lyt-2$^+$ lymphocytes remained inconclusive because the total number of positive cells (L3T4$^+$ plus Lyt-2$^+$) enumerated by indirect immunofluorescence did not add up to the number of Thy-1$^+$ T cells.

Effect of anti-L3T4, anti-I-A, and anti-Lyt-2 MA treatment on SEA- and ConA-induced blastogenesis of splenic lymphocytes. At the time that antibody-treated, infected mice were sacrificed and their livers removed for granuloma measurement, single-cell suspensions were made of three to four separate spleens of mice from each group. Cells were stimulated with either ConA or SEA, and their lymphoproliferative responses were assayed by [^{3}H]TdR uptake. SEA-induced proliferation was suppressed in three of four spleens, although as a group there was no statistical difference when thymidine uptake was compared with that in the saline-treated control group (Table 2). Anti-I-A and anti-Lyt-2 did not affect the antigen-induced blastogenesis. ConA-induced proliferation was not affected in either anti-L3T4- or anti-I-A-treated mice, whereas anti-Lyt-2-treated animals demonstrated a significant 37% increase ($P < 0.05$).

Antigen-induced IL-2 production of splenic cells suppressed by anti-L3T4 MA treatment. To elucidate the mechanisms of the remarkable granuloma suppression by anti-L3T4 MA, the IL-2 production of these animals was assayed. Spleen cells from antibody-treated animals used for the granuloma studies were stimulated with SEA, and the supernatants were assayed for IL-2 activity (Fig. 3). Whereas after SEA stimulation splenic lymphocytes of saline (control)-treated animals produced more than 32 U of IL-2 per ml, spleen cells from anti-L3T4 MA-treated mice did not release any detectable amount of IL-2 (<0.01 U/ml). Splenocytes from anti-I-A MA-treated animals also showed significant suppression of IL-2 production. In contrast, anti-Lyt-2 MA-treated animals produced IL-2 levels comparable to those of saline-treated animals. [^{3}H]TdR incorporation by CTLL20 cells stimulated with undiluted supernatants of SEA-stimulated splenic lymphocytes was also determined. Spleen cells of anti-L3T4 MA-treated mice appeared to produce very low amounts of IL-2 that induced weak [^{3}H]TdR uptake by the cell line. This low level of IL-2 was insufficient for calculating IL-2 units by probit analysis.

DISCUSSION

Previous work established that the Lyt-1$^+$ subset of splenic T_{DH} cells is responsible for the adoptive transfer of granulomatous response and migration inhibitory factor (MIF)-active lymphokine production (6, 26). This was corroborated in an in vitro model of schistosome egg-induced granuloma formation (11). Moreover, a T-cell line established from liver granuloma lymphocytes that adoptively transferred granulomas comprised predominantly Lyt-1$^+$ and L3T4$^+$ T cells (Ragheb et al., 1985). Finally, SEA-specific L3T4$^+$ T-cell clones were shown to augment in vitro granuloma formation around SEA-coupled beads (18).

Based on this profile of inflammatory T_{DH} lymphocytes, it was anticipated that prolonged administration of anti-L3T4 MA to *S. mansoni*-infected mice would affect granuloma formation. The results clearly indicate that such treatment given between 6 and 8 weeks after infection indeed resulted in a dramatic 68% suppression of the hepatic granulomatous inflammation.

TABLE 2. Blastogenic response of spleen cells from infected animals

Treatment	Spleen no.	[^{3}H]TdR incorporation (Δcpm)			
		SEA		ConA	
		Mean[a]	Group mean ± SEM	Mean[a]	Group mean ± SEM
Saline (control)	1	11,050	13,090 ± 2.547	75,133	82,493 ± 9,034
	2	18,154		60,936	
	3	10,068		92,346	
	4	—[b]		101,557	
Anti-L3T4 MA	1	3,689	6,135 ± 1,744	76,934	66,869 ± 4,233
	2	6,392		60,912	
	3	3,482		70,727	
	4	10,976		58,905	
Anti-I-A^k MA	1	14,949	11,475 ± 1,860	55,488	62,699 ± 4,449
	2	10,893		56,803	
	3	8,584		63,557	
	4	—		74,949	
Anti-Lyt-2 MA	1	7,863	12,966 ± 2,775	111,686	133,080 ± 7,241
	2	12,628		99,302	
	3	17,409		133,364	
	4	—		107,969	

[a] Means of quadruplicate cultures.
[b] —, Not determined.

Anti-L3T4 MA treatment of infected mice caused a fourfold decrease in the number of L3T4$^+$ splenic T cells compared with control saline-treated animals. Although the relative percentage of T cells actually increased within the granulomas of anti-L3T4 MA-treated mice, it is inferred that due to the drastic reduction in lesion size, the absolute number of intragranulomatous Thy-1.2$^+$ cells diminished.

The suppression of the granuloma could have resulted from at least three mutually nonexclusive factors: (i) blockade of T_{DH} cell function by the MA; (ii) reduction in the total number of available T_{DH} cells; and (iii) imbalance between the ratio of T_{DH} and T suppressor lymphocytes within the granulomas.

The first alternative is strongly corroborated by the finding of elevated proportions of T lymphocytes in the diminished granulomas. It is envisioned that anti-L3T4 MA blocked the function of the intragranulomatous T cells and inhibited IL-2 production. This premise is supported by data showing virtual cessation of antigen-induced IL-2 production by splenic cells of anti-L3T4 MA-treated mice. This could occur by inhibition of class II MHC determinant binding by L3T4$^+$ T cells (14) or interference with signal transmission in these lymphocytes (25).

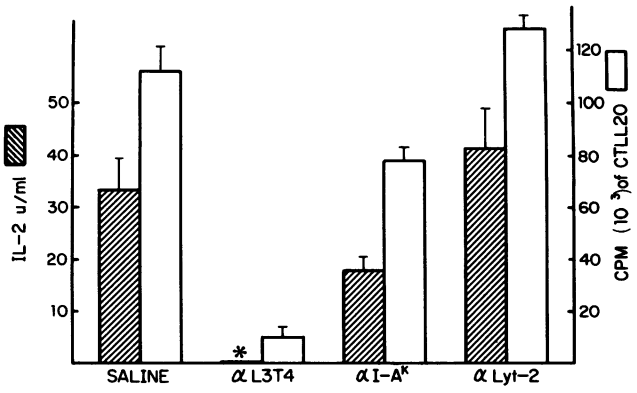

FIG. 3. SEA-induced IL-2 production (hatched bars) by spleen cells from animals treated with MAs. Open bars indicate the [^{3}H]TdR uptake by CTLL20 cells cultured with undiluted supernatant. Asterisk indicates IL-2 production < 0.01 U/ml. Bars represent the mean ± standard error for four spleens.

Anti-L3T4 MA added to cultures of antigen-stimulated T-cell clones blocked IL-2 and gamma interferon production (28). We assume that in our system, blockade of IL-2 production also caused impaired inflammatory lymphokine, notably MIF and eosinophil stimulation promoter secretion. Insufficient local production of these lymphokines (3, 17) would explain the formation of smaller granulomas. This idea was substantiated by histopathological observations of liver tissues. Few eosinophils and mononuclear cells, immature macrophages, and minimal or absent collagen deposition were seen in the diminished lesions. These immature granulomas may not efficiently sequester the secretions of the schistosome eggs. Absence of adequate sequestration in turn would allow some cytotoxic damage to hepatocytes that are adjacent to the granulomas. Although the damage we observed was not pronounced, the histopathology of the liver resembled that seen in schistosome-infected athymic (nude) mice (5). Thus, L3T4$^+$ T_{DH} effector cells may be crucial in cellular recruitment, macrophage differentiation and activation, epithelioid and giant cell formation, and direct (19) or macrophage-mediated (29) fibroblast activation.

The second alternative that considers a paucity in the total number of available T_{DH} cells after anti-L3T4 MA treatment is illustrated by the greatly diminished absolute T lymphocyte counts found in the spleens of granulomas of the treated animals. Because anti-L3T4 MA blocked IL-2 production, it is plausible that in the absence of such lymphokine, clonal expansion of antigen-specific T_{DH} cells is severely curtailed. The last alternative, imbalance between effector and suppressor T lymphocytes, is also an important consideration, which will be investigated in a subsequent study.

Elimination of L3T4$^+$ T cells may also have therapeutic effects. Reduction of this subset by in vivo-administered anti-L3T4 MA inhibited the progression of cutaneous leishmaniasis in genetically susceptible mice (23), prevented

paralysis during the development of EAE (24), and arrested the occurrence of renal disease in NZB/NZW mice (28). A future study should establish whether in the schistosomiasis model the diminished granulomas and fibrosis with slight hepatic damage would have a moderating effect on the pathophysiology of the disease.

In contrast to anti-L3T4 treatment, injections of anti-I-A MA did not suppress the granulomatous response, but significantly diminished antigen-induced IL-2 production. Because granuloma macrophages of *S. mansoni*-infected mice were shown to present SEA to T lymphocytes in the context of both I-A and I-E subregion class II MHC antigens (12), it is possible that after I-A antigen blockade the I-E subregion-encoded antigens were sufficient for elicitation of IL-2 production and maintenance of the normal granulomatous response. In the autoimmune EAE model, anti-I-A MA treatment of mice did prevent mortality and reduced relapses (22), although anti-L3T4 MA treatment evoked a more pronounced therapeutic effect (24).

Finally, administration of anti-Lyt-2 MA diminished the number of Lyt-2^+ cells in the spleens, but apart from raising [^{3}H]TdR incorporation in ConA-stimulated splenic cells, it had no effect on either IL-2 production or granuloma formation. This was not unexpected because the anti-Lyt-2 MA used was not cytotoxic. Although treatment with the antibody appeared to diminish the number of Lyt-2^+ T cells, the rather weak T suppressor activity in the acutely infected mice was not altered.

The present study demonstrates that T_{DH}-cell-mediated IL-2 production and granuloma formation are correlated. This observation is consonant with clinical data showing that the high-intensity alveolitis that precedes sarcoid granuloma formation and impaired granuloma formation in lepromatous leprosy patients were correlated with enhanced or diminished IL-2 production by patient T_H lymphocytes (15, 16). Thus, the major prerequisites for adequate granulomatous response are sufficient IL-2 production, clonal expansion of T effector lymphocytes, and secretion of inflammatory lymphokines.

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