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Identification of Larval Cross-Reactive and Egg-Specific Antigens Involved in Granuloma Formation in Murine Schistosomiasis Mansoni

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Cross-reactive humoral immune responses between antigens of different developmental stages of the worm *Schistosoma mansoni* have previously been demonstrated. In contrast, information on antigenic cross-reactivity at the T-cell level is still very sparse. The present study examined the cross-reactive T-cell responses to eggs and crude and fractionated soluble egg antigens (SEA) in infected mice prior to (from 0 to 4 weeks of infection) and after (5 weeks and onwards) egg deposition. Splenic lymphocyte proliferation to unfractionated SEA was detected as early as 2 weeks postinfection and increased rapidly by 4 weeks postinfection. Injections of live eggs into the lungs of infected mice at 4 weeks postinfection demonstrated enhanced granuloma formation, indicating the presence of primed T cells that respond to egg antigens. Further experiments with the artificial granuloma model and polyacrylamide gel electrophoresis-separated SEA fractions demonstrated that in mice infected for 4 weeks the 60- to 66-, 93- to 125-, and >200-kDa SEA fraction-coated beads elicited significant pulmonary granulomas. By 6 weeks postinfection, when eggs are deposited in the livers, in addition to the cross-reactive fractions (60 to 66, 93 to 125, and >200 kDa), beads coated with fractions of 25 to 30, 32 to 38, and 70 to 90 kDa also elicited significant granulomatous reactions. These antigenic fractions are considered to have elicited egg stage-specific T-cell responsiveness. In addition hepatic granuloma T cells from the 6th week of infection demonstrated the strongest blastogenic response to the 60- to 66-kDa cross-reactive fraction. Thus, in vitro and in vivo experiments demonstrated T-cell cross-reactivity between the larval and egg stages of the worm. On the basis of these observations, the appearance of the primary circumovum granulomatous response in infected mice is considered to represent the sum of larval cross-reactive and egg-specific T-cell responsiveness.

Schistosoma mansoni worms cause a chronic granulomatous disease in the tropics (3). The pathology observed in schistosomiasis mansoni is caused by the chronic T-cell-mediated granulomatous inflammations and fibrosis around disseminated eggs (3, 25, 29). In mice, eggs are produced and released 5 to 5 1/2 weeks postinfection. After the induction of T-cell-mediated immunity by soluble egg antigens (SEA) (5), the acute phase of the disease occurs from 8 to 10 weeks and is characterized by vigorous granuloma formation (3, 25). By 16 to 20 weeks, the chronic phase of the disease begins and the intensity of the inflammatory response is down-modulated (4, 7) by subsets of suppressor T cells (6, 8, 15, 26) and their soluble factors (1, 17, 24).

Recent studies in our laboratory have delineated the T-cell responsiveness of infected mice to fractionated SEA antigens during the acute and chronic stages of the infection (22). Results demonstrated a change in the pattern of specific T-cell lymphoproliferative responses that accompany the down-modulation of the granulomatous response. The present studies were initiated to examine T-cell responsiveness at the preoviposition stage of the infection. Whereas earlier studies have demonstrated antibody cross-reactivity (2, 16, 18, 30, 31) to egg, cercarial, and schistosomular antigens, cross-reactive T-cell responses have been sparsely documented (21, 23).

This study was designed to examine in infected mice cross-reactive granulomagenic T-cell responses to eggs, SEA, and SEA protein fractions prior to and immediately subsequent to egg release. The data demonstrate that some SEA fractions are cross-reactive with the schistosomula developmental-stage antigens, while others are egg specific. Larval antigens that share epitopes with SEA presensitize the system and thus enhance the SEA-specific egg granuloma response.

MATERIALS AND METHODS

Mice. Female CBA/J ($H\text{-}2^k$) mice were purchased from Jackson Laboratories, Bar Harbor, Maine. The mice were maintained under standard laboratory conditions.

Infection. Six-week-old mice were infected subcutaneously with 25 cercariae of the Puerto Rican strain of *S. mansoni* and examined at 4 and 6 weeks postinfection.

Preparation of SEA. SEA were prepared from eggs isolated from the livers of mice infected with 200 cercariae (5).

SEA separation and fractionation. Crude SEA were fractionated as previously described (22). The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide)-separated SEA proteins were divided into nine fractions, <21, 25 to 30, 32 to 38, 40 to 46, 50 to 56, 60 to 66, 70 to 90, 93 to 125, and >200 kDa, according to molecular mass. The pooled fractions were electroeluted (Bio-Rad, Richmond, Calif.) from the gel slices, dialyzed in phosphate-buffered saline, filter-sterilized, assayed for protein content, and adjusted to 6 to 8 μg/ml. The fractions were then used in T-cell blastogenic assays in vitro and granuloma elicitation in vivo.

Spleen cell preparation. Single-cell suspensions were prepared from spleens at 1, 2, 3, and 4 weeks after infection.

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Erythrocytes were lysed with Tris-ammonium chloride (pH 7.2), and the remaining cells were suspended in RPMI 1640 and counted with trypan blue vital dye. The spleen cells from mice infected for 4 weeks were further separated on nylon wool, and the nylon wool-nonadherent (NWN) T-cell-enriched population (19) was used in blastogenic assays.

Granuloma T-cell isolation. Hepatic granuloma T cells were obtained from perfused livers as previously described (26). At 6 weeks postinfection, the plastic plate-nonadherent fraction of the granuloma cells consisted of 50 to 55% eosinophils, 5 to 10% macrophages, and 15 to 20% lymphocytes.

Blastogenic assays. Whole-spleen cell populations from mice infected for 1, 2, 3, and 4 weeks, NWN splenic T cells from mice infected for 4 weeks, and granuloma cells from mice infected for 6 weeks were cultured in 96-well microtiter plates at 1×10^6 cells per ml for spleen or 2×10^6 cells per ml for granuloma cells in RPMI 1640 supplemented with 5% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.), 5×10^{-5} M 2-mercaptoethanol (Eastman Organic Chemical, Rochester, N.Y.), 2 mM sodium pyruvate, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin (M. A. Bioproducts, Walkersville, Md.) per ml. The SEA (10 μg/ml) or SEA fractions (4 to 6 μg/ml) to be tested were added to the wells at 50 μl per well, and the total volume was raised to 200 μl per well with medium. The negative controls consisted of a blank elution and a medium control. After 4 days of culture, the cells were pulsed for 12 to 16 h with 1 μCi of [^{3}H]thymidine (ICN Radiochemicals, Irvine, Calif.), harvested onto glass wool filters (type A/E; Gelman Sciences, Inc., Ann Arbor, Mich.), and measured for radioactivity in a liquid scintillation counter. Data are represented as the change in counts per minute versus the blank control. Because preliminary experiments demonstrated high backgrounds with the normal 4-day blastogenic assay, the 6-week-postinfection granuloma cells were allowed to rest for 3 days before the blastogenic culture.

Preparation of SEA fraction-coated beads. Antigen-coated beads were prepared by coupling the SEA fractions to cyanogen bromide-activated Sepharose 4B beads (Pharmacia, Piscataway, N.J.) sieved to obtain a diameter between 40 and 80 μm. The pooled fractions of SEA were concentrated to 30 to 35 μg of protein per ml prior to coupling by aquacide dialysis using dialysis tubing with a 12- to 14-kDa exclusion limit. The SEA protein fractions were dialyzed against 0.1 M sodium bicarbonate, pH 8.3, containing 0.5 M NaCl. A ratio of 30 μg of protein per 50,000 beads was coupled at 4°C overnight. Unreacted sites were blocked by the addition of 1 M ethanolamine-HCl, pH 8, for 2 h at 4°C.

Measurement of lung granulomas elicited by *S. mansoni* eggs or SEA fraction-coated beads. Two thousand live, mature *S. mansoni* eggs or SEA fraction-coated beads were injected into the tail veins of mice at 4 weeks postinfection. After 2 days (in the case of the eggs) or 4 days (in the case of the beads), animals were sacrificed and their lungs were inflated, preserved with phosphate-buffered formalin (Fisher, Springfield, N.J.), sectioned, and stained with hematoxylin and eosin. The areas of the granulomas were measured by means of computer image analysis with computer analysis software (The Microscope Co., Medina, Ohio) and expressed as area in square micrometers. Only those lesions around beads with diameters ranging between 40 and 75 μm were measured.

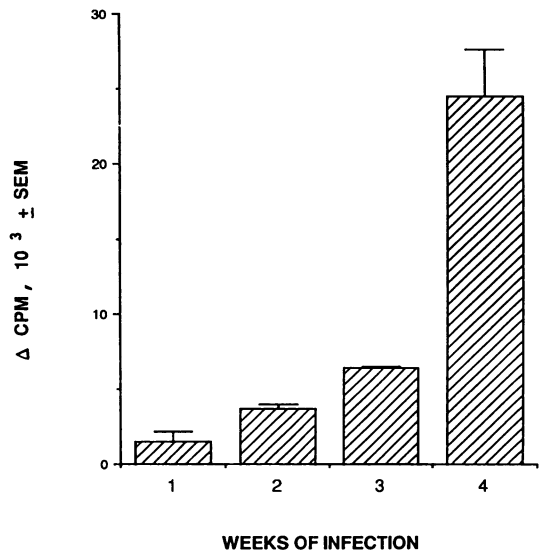

FIG. 1. Splenic lymphocyte blastogenic response to crude SEA of mice infected for 1, 2, 3, and 4 weeks. Background proliferation for unstimulated spleen cells ranged from 2,000 cpm at 1 week postinfection to 5,500 cpm at 4 weeks postinfection. Data represent the means of two experiments. In each experiment, spleens pooled from at least three mice were used.

Statistical analysis. The unpaired Student *t* test was used to examine the granuloma elicitation and blastogenic data. Data were determined to be significant at $P < 0.05$.

RESULTS

Examination of SEA reactivity of splenic lymphocytes prior to egg release. Earlier literature has demonstrated the presence of cross-reactive T-cell epitopes on schistosomula and egg antigens (21, 23). Therefore the kinetics of cross-reactive T-cell responses to SEA were examined between weeks 1 and 4 of the infection. Figure 1 demonstrates that 1 week postinfection virtually no SEA-specific proliferative reactivity was present. However, by weeks 2 to 3 postinfection, significant increases in anti-SEA reactivity were demonstrated (differences of 4,100 and 6,440 cpm, respectively), and by 4 weeks postinfection further enhancement was seen (a difference of 24,326 cpm).

Identification of SEA fractions that elicit cross-reactive T-cell blastogenic responses. To determine those SEA fractions that elicit cross-reactive T-cell response in vitro, NWN splenic T cells of mice at 4 weeks postinfection were used in blastogenic assays. Figure 2 illustrates that the fractions of 25 to 30, 60 to 66, 93 to 125, and >200 kDa of SEA elicited significant cross-reactive ($P < 0.05$) blastogenic responses. The fractions of <21, 32 to 38, 40 to 46, 50 to 56, and 70 to 90 kDa did not elicit significant proliferative responses.

Anamnestic lung granuloma formation in mice at 4 weeks postinfection. To determine whether the strong SEA-cross-reactive splenic T-cell responses are relevant to granuloma formation, live eggs were injected into the lungs of uninfected mice and of mice infected for 4 weeks. Compared with mean granuloma sizes in the lungs of naive mice, the enhanced, accelerated granulomatous response that appeared day 2 postinfection indicated an anamnestic response ($P < 0.001$) in mice at 4 weeks postinfection (Fig. 3).

Identification of SEA fractions that elicit anamnestic, cross-

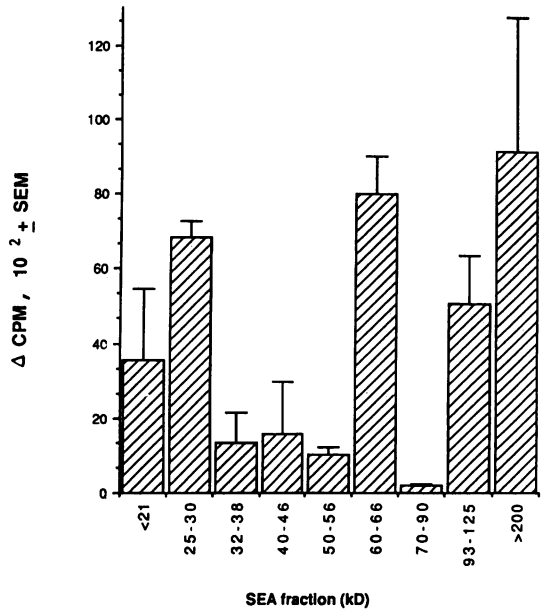

FIG. 2. Splenic T-cell response of mice infected for 4 weeks to SDS-PAGE separated SEA fractions. Background proliferation of unstimulated splenic T cells was 5,620 cpm. Data depict a representative experiment with each fraction tested in triplicate. Two repeat experiments showed similar results. In each experiment, spleens pooled from at least three mice were used.

reactive granulomatous responses. To examine more specifically the SEA fractions responsible for granuloma formation, fractions containing identical amounts (30 μg) of protein were covalently coupled with beads, and the beads were injected intravenously (i.v.) into the lungs of mice infected for 4 weeks. Figure 4 shows those fractions which elicited a granulomatous response. The SEA fractions of 60 to 66, 93 to 125, and >200 kDa elicited significant ($P < 0.001$) granulomas around the coated beads, whereas the fractions of <21, 25 to 30, 32 to 38, 40 to 46, 50 to 56, and 70 to 90 kDa did not elicit an inflammatory response.

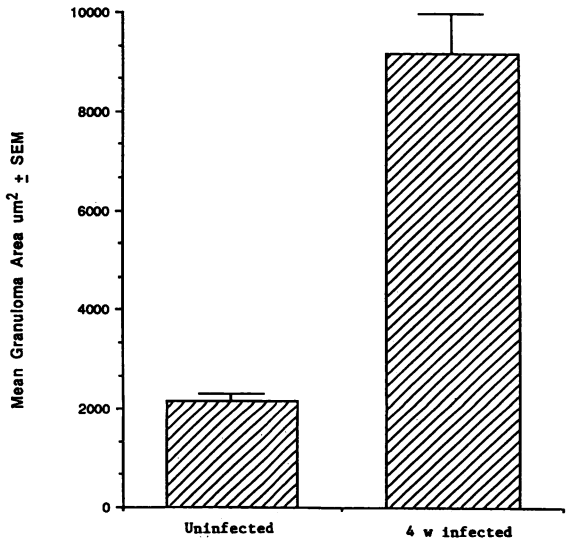

FIG. 3. Pulmonary granuloma formation in normal and in mice infected for 4 weeks. Mice were injected i.v. with 2,000 viable eggs in a volume of 500 μl. Two-day-old granulomas were measured in stained histologic sections. Data represent the means of 100 granuloma measurements, with five mice in each group.

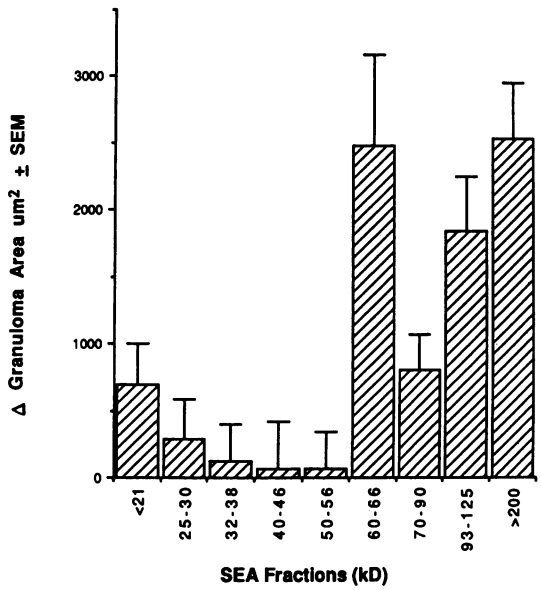

FIG. 4. Pulmonary granuloma formation around SEA fraction-coated beads in mice infected for 4 weeks. Mice were injected i.v. with 2,000 SEA fraction-coated beads in a volume of 500 μl. Four-day-old granulomas were measured in stained histological sections. Data represent the differences between the means and the values for the bovine serum albumin (BSA)-coated control beads of at least 100 granuloma measurements, with five to six mice in each group. The mean area of the BSA-coated control bead granulomas was 6,700 ± 327 μm^2.

Identification of SEA fractions that elicit egg-specific responses. To determine whether the SEA-cross-reactive granulomatous response undergoes a change after egg deposition, fraction-coated beads were injected i.v. into mice infected for 6 weeks. The results depicted in Fig. 5 show that the SEA fractions of 25 to 30, 32 to 38, and 70 to 90 kDa also elicited significant ($P < 0.001$) responses, whereas the intensity of responses observed at week 4 postinfection to the fractions of 60 to 66, 93 to 125, and >200 kDa remained unchanged. For comparison, unseparated SEA-coated bead lung granulomas in mice at week 8 postinfection showed an increase in area over the bead control of 3,300 ± 958 μm^2. These beads presumably would have a mixture of granuloma-eliciting and noneliciting antigens from crude SEA bound to their surfaces.

To further identify the egg-specific SEA fractions, we sensitized naive mice with an intraperitoneal injection of 2,000 live eggs and examined splenic T-cell responses to SEA fractions at intervals. Blastogenic responses at 4 days postimmunization showed reactivity to only the 25- to 30- and 32- to 38-kDa fractions (differences of 4,200 ± 463 and 9,550 ± 1,739 cpm, respectively). Responses at 16 days postimmunization were observed to all nine SEA fractions, with difference values ranging from 3,500 to 7,500 cpm.

Granuloma T-cell blastogenic responses to SEA fractions of mice at 6 weeks postinfection. Examination of granuloma T-cell blastogenic responses of mice at 6 weeks postinfection to the nine SEA fractions allowed the characterization of focal T-cell responses that exist during the development of

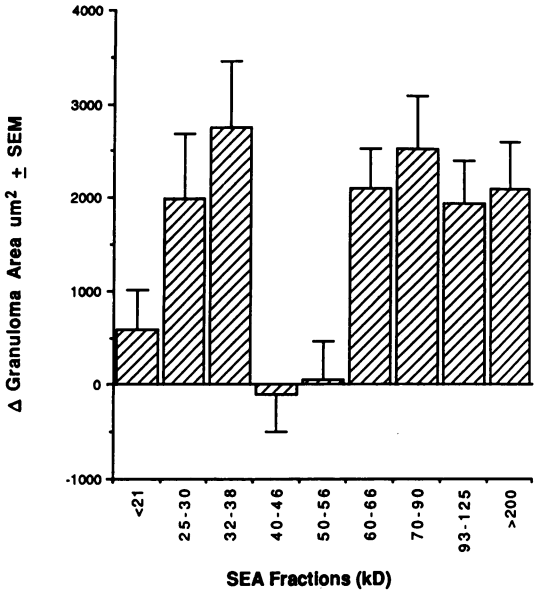

FIG. 5. Pulmonary granuloma formation around SEA fraction-coated beads in mice infected for 6 weeks. Experimental conditions are identical with those shown in Fig. 4. The mean area of the BSA-coated control bead granulomas was 7,100 ± 311 μm^2.

the hepatic granulomas. Figure 6 illustrates that the 60- to 66-kDa fraction, which was shown to belong to the cross-reactive antigens (Fig. 4), elicited the highest proliferative response (a difference of 4,523 cpm) ($P < 0.05$). The fractions of <21, 25 to 30, 32 to 38, 40 to 46, 50 to 56, 70 to 90, 93 to 125, and >200 kDa gave lower, nonsignificant proliferative responses.

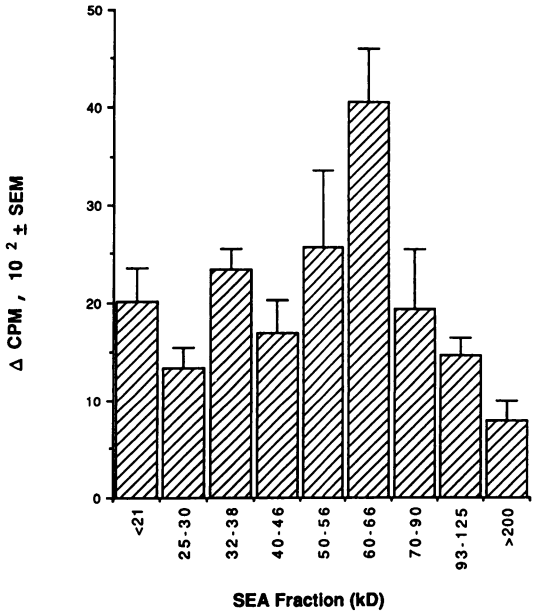

FIG. 6. Granuloma cell blastogenic responses to SEA fractions at 6 weeks postinfection. Background proliferation in the three experiments ranged from 2,200 to 4,500 cpm and was subtracted from the mean of each group. Data represent the means of three experiments. In each experiment, granuloma cells were pooled from at least five livers.

DISCUSSION

In this study, we present evidence that T cells sensitized to *S. mansoni* larval antigens during the preoviposition period (0 to 4.5 weeks) of infection can respond to egg antigens. This was demonstrated in mice infected for 4 weeks by the strong proliferative responses of splenic lymphocytes to unseparated SEA and the enhanced pulmonary circumovum granuloma formation. Moreover, to identify larval-egg cross-reactive and egg-specific antigenic groups, SDS-PAGE-separated SEA fractions and the artificial, bead pulmonary granuloma model were used before and after egg deposition. The SEA fractions that elicited significant proliferative and granulomatous responses at 4 weeks postinfection (prior to egg deposition) included the fractions of 60 to 66, 93 to 125, and >200 kDa (Fig. 4). These fractions are identified as being cross-reactive and sharing epitopes with larval antigens. After egg deposition and sensitization to egg antigens, the mice infected for 6 weeks developed significant granulomatous responses around beads coated with the cross-reactive SEA fractions (Fig. 4) but also responded to additional fractions (25 to 30, 32 to 38, and 70 to 90 kDa). We infer from the pattern of this responsiveness that these three fractions are egg specific. This contention is supported by the splenic cell proliferation data of egg-sensitized mice, which showed the earliest response to the 25- to 30- and 32- to 38-kDa fractions of SEA. The pattern of granulomatous responsiveness to SEA fractions at 8 weeks postinfection did not differ from that of mice infected for 6 weeks (data not shown).

The foregoing results demonstrate that the early hepatic granulomatous response that appears around the freshly deposited eggs at 5 to 6 weeks postinfection represents a composite larval cross-reactive and egg-specific T-cell reactivity. Indeed, T lymphocytes isolated from the developing hepatic granulomas of mice infected for 6 weeks stimulated with the 60- to 66-kDa fraction also showed proliferation to cross-reactive antigens.

The existence of SEA cross-reactive humoral immune responses prior to egg deposition is well documented. Early studies with immunoprecipitation demonstrated multiple epitopes shared between eggs and cercariae and between eggs and adult worms (28). Antibodies from egg-immunized mice were shown to react with mechanically transformed schistosomula (2). Conversely, antibodies from schistosomula-immunized mice reacted with egg glycoproteins (9). More specifically, an anti-egg monoclonal antibody showed surface binding to a >200-kDa schistosomula protein (18) and provided partial protection to mice against cercarial challenge. An antischistosomula monoclonal antibody which recognized a >200-kDa schistosomula antigen and reacted with miracidial antigens has also been reported (31). In contrast, there is a scarcity of data on T-cell cross-reactivity between larval and egg antigens. Early publications found a lack of anamnestic granulomatous responsiveness to i.v. injected eggs in mice infected for 2 weeks or in mice immunized with irradiated cercariae (14, 29). In contrast, lymphocytes from the draining lymph nodes of mice infected for 1 week were shown to form rosettes around SEA-coated erythrocytes (20) and proliferate under SEA stimulus (21). The latter observations were confirmed in the present experiments, though the splenic cells showed reactivity only from the second week of the infection and onwards. Splenic T-cell

clones of infected mice also showed SEA-specific as well as SEA and cercarial, schistosomular antigen cross-reactive proliferation (23). The presence of SEA cross-reactive T cells in the draining lymph nodes versus the lack of granulomatous responsiveness of mice during the early stages of infection is best explained by numerical considerations; during the first weeks of the infection there may be an insufficient number of recirculating memory T cells available to enter the lung and initiate a circumovum granulomatous response. This capacity is clearly acquired by the fourth week of infection, when both strong proliferative and granulomagenic responses are observed (Fig. 1, 2, 3, and 4). Alternatively, because splenic T-cell reactivity to SEA is most pronounced after 3 to 4 weeks postinfection (Fig. 1), the late stage of the developing schistosomulae may provide the antigenic epitopes that induce the cross-reactive T-cell responsiveness with egg antigens.

Several important implications may arise from the presently described T-cell cross-reactivity. During superinfection, the granulomatous immune response engendered by eggs in the lungs has been suggested to contribute to resistance by the elimination of migrating schistosomula (10, 12, 13). Thus, cross-reactive fractions of egg antigens may contribute to antischistosomular resistance during superinfection. Conversely cross-reactivity at the T-cell level between larval and egg antigens may influence the evolution and intensity of the granulomatous response (10, 11). In the present experiments, presensitization to egg antigens by cross-reactive larval antigens clearly enhanced the subsequent circumovum granulomatous response.

This study identified antigenic fractions of SEA that share epitopes with larval antigens and also those that appear to be egg specific. T-lymphocyte populations sensitized to the different groups of antigens together contribute to the development of the egg-induced granulomas. This information should be valuable for the development of vaccines (27) prepared against the schistosomula stage in that it may help eliminate the use of those antigens that can prime for an enhanced granulomatous response. The availability of purified antigens that specifically induce circumovum granulomatous responses may enable the development of an "antipathology vaccine" that would ameliorate the intensity of the granulomatous inflammatory response and alleviate the pathology of the disease.

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