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# LOW MOLECULAR WEIGHT EOSINOPHIL CHEMOTACTIC FACTOR IN GRANULOMATOUS LIVER OF MURINE SCHISTOSOMIASIS[1]

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**An acidic peptide, preferentially chemotactic for eosinophils, was extracted from livers of mice infected with *Schistosoma mansoni*. Sephadex G-25 column chromatography showed that the majority of the eosinophil chemotactic activity was detected in the fractions just after elution of the molecular marker vitamin $B_{12}$ (m.w. 1355.4). This activity began to appear in the livers of some mice 5 weeks after infection. Peak activity was detected at 8 to 12 weeks after infection and persisted at least until 16 weeks. It was sensitive to carboxypeptidase-A. By Dowex-1 anion exchange chromatography, the activity eluted as a narrow peak at pH 3.1 to 2.6 as shown for eosinophil chemotactic factor of anaphylaxis (ECF-A). The activity was also detected in a broad peak at pH 6.3 to 3.7. Unlike ECF-A, the activity was stable to boiling in both acid and alkali. These findings suggest that granulomatous liver of murine schistosomiasis-derived eosinophil chemotactic factor (ECF-G) may play a specific role in eosinophil accumulation in this chronic inflammation.**

Schistosomiasis manifests eosinophilia in man (1) and experimental animals (2–4), but the mechanisms for this are not completely understood. Two chemotactic factors for eosinophils, eosinophil stimulation promotor (ESP)[2] and diffusible stimulator of eosinophilopoiesis, have been reported to exist in mice infected with *Schistosoma mansoni* (*S. mansoni*). ESP is a lymphokine, produced *in vitro* by incubation of splenic cells from the infected mice with soluble antigenic substances of *S. mansoni* eggs (5). It shows chemotactic activity for both monocytes and eosinophils *in vitro* (6). The diffusible stimulator of eosinophilopoiesis was initially described by Miller and McGarry (7) in tetanus toxoid-sensitized mice as derived from antigen-stimulated lymphoid cells. Miller, Colley, and McGarry (8) demonstrated that lymphoid cells of *S. mansoni*-infected mice produce a similar stimulator of eosinophils.

Recently, Phillips and Colley (9) reviewed the literature concerning the relationship of eosinophils to schistosomiasis. They pointed out the possibility that eosinophil chemotactic factor of anaphylaxis (ECF-A) (10) might also be involved in schistosomiasis.

In this study, we have extracted and partially purified an eosinophil chemotactic factor from livers of mice infected with *S. mansoni*. The peptide-like factor obtained was compared with ECF-A for its biochemical and biologic properties.

## MATERIALS AND METHODS

*Animals and method of infection.* Young adult male mice of the BALB/c strain (18 to 22 g weight) were used. Groups of 30 to 50 mice were infected by subcutaneous injection of 50 cercariae of the Puerto Rican strain of *S. mansoni* freshly hatched from snails.

*Extraction and partial purification of granulomatous liver-derived chemotactic factor.* Livers were removed at different times 4 to 16 weeks after infection and stored at −70°C until used. Age-matched normal mice were sacrificed to obtain control livers. One gram wet weight of each liver was minced, washed twice in 0.9% NaCl (saline), suspended in 5 ml saline, and homogenized by glass homogenizer at 4°C. The homogenate was centrifuged at 20,000 × G for 15 min at 4°C. The supernatant was then put in Spectra/por 3 membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.) to cut the m.w. at 3500 and dialyzed against 100 times volume of RPMI 1640 medium (Grand Island Biological Co., Grand Island, N. Y.) containing 100 units/ml of penicillin and 100 μg/ml of streptomycin for 24 hr at 4°C. The diffusate as well as the nondialyzable residue were assayed for eosinophil chemotactic activity after adjusting to pH 7.4.

For partial purification, livers of mice after 8 weeks of infection were homogenized in saline and dialyzed against water (100 × v/v) with Spectra/por 3 membrane. The diffusate was lyophilized and reconstituted to the original sample volume by cold water; 1.5 ml of these samples were applied to a Sephadex G-25 fine (Pharmacia Fine Chemicals, Piscataway, N. J.) column (1.6 x 92.6 cm) pre-equilibrated with 0.067 M phosphate buffered-saline (PBS), pH 7.4. The column was eluted with PBS and the fractions collected (2.5 ml/tube) were tested for eosinophil chemotactic activity after dilution with RPMI 1640 medium (20 × v/v). Blue dextran (m.w. $2 \times 10^6$, Pharmacia, Uppsala, Sweden), vitamin $B_{12}$ (m.w. 1355.4, Sigma Chemical Co., St. Louis, Mo.), and L-tyrosine (m.w. 181.19, Nutritional Biochemicals Corp., Cleveland, Ohio) were used for calibration.

*Measurement of chemotaxis in vitro.* Peritoneal exudates rich in eosinophils were prepared by weekly injection of Hartley strain guinea pigs with 2 ml of horse serum (Grand Island Biological Co.) for 8 to 12 weeks after a modification of the method of Litt (11). Peritoneal cells containing 50 to 80%

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[2] Abbreviations used in this paper: ESP, eosinophil stimulation promotor; *S. mansoni, Schistosoma mansoni*; ECF-A, eosinophil chemotactic factor of anaphylaxis; hpf, high power fields; ECF-G, granulomatous liver of murine schistosomiasis-derived eosinophil chemotactic factor; ECF-C, complement-dependent eosinophil chemotactic factor.



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eosinophils were collected in Hanks' balanced salt solution (Flow Laboratories, Rockville, Md.) containing 10 units/ml of sodium heparin (Organon, Inc., West Orange, N. J.) on the 5th day after the last injection. The cells were centrifuged (200 × G), washed twice, and resuspended in RPMI 1640 medium containing 0.5% five-times crystallized ovalbumin (Sigma) at a concentration of $5 \times 10^5$ cells/ml.

Eosinophil chemotactic activity was quantified by a modification of Boyden's Method (12). Blind-well chemotactic chambers (Nuclepore Corp., Pleasanton, Calif.) and polycarbonate membrane filters with 5.0-μg pores (Nuclepore) were used. They were filled with 0.75 ml of eosinophil-rich cell suspension in the upper compartment and 0.2 ml dialyzed liver extracts, fractions of Sephadex G-25 column chromatography, positive chemotactic stimulus, or RPMI 1640 medium (spontaneous eosinophil locomotion) in the lower compartment. The chambers were incubated for 90 min at 37°C in a 5% $CO_2$-95% air environment. The filters were then fixed in $HgCl_2$-ethanol solution for 15 hr and stained with Carrazzi's haematoxylin and Chromotrope 2R (K & K Laboratories, Inc., Jamaica, N. Y.) (13). Each liver extract was tested in triplicate. The number of cells migrating from the upper side to the lower side of the filter was counted in 10 high power fields (hpf) (10 × 40) randomly selected, and an average number was calculated to obtain migrated cells per field. Chemotactic activity was expressed as an average of the migrated cells per hpf in three filters for each animal.

For positive chemotactic stimulus, guinea pig serum activated with 2 mg/ml of Zymosan A (Lot No. 106C-0352, Sigma) at 37°C for 1 hr was used (14). The Zymosan A was removed by centrifugation and the serum was heated to 56°C for 30 min to terminate the reaction. The serum was diluted ten times with RPMI 1640 medium and placed in the lower compartment.

In separate experiments, reliability of chemotactic activity was tested by filling the upper compartment with eosinophil-rich cell suspension and adding 0.2 ml or 0.75 ml of dialyzate of the liver obtained 8 weeks after infection to both upper and lower compartments.

*Effects of carboxypeptidase A, boiling, acidification, and alkalization.* The fractions from Sephadex G-25 column chromatography that showed the major eosinophil chemotactic activity were pooled, lyophilized, and redissolved in 2 ml cold distilled water. Carboxypeptidase-A (Lot No. 48C-8060, Sigma) was mixed at a concentration of 300 units/ml and incubated at 37°C for 3 hr. The reactant was dialyzed with Spectra/por 3 membrane against 100 times volume of RPMI 1640 medium at 4°C for 24 hr. The pooled fraction with chemotactic activity was treated with heated acid and alkali as described by Baba *et al.* (15).

*Purification by Dowex-1 anion exchange chromatography.* Further purification of this activity by Dowex-1 anion exchange chromatography was performed according to the method of Boswell *et al.* (16). Livers of mice after 8 weeks of infection were homogenized in saline and dialyzed against water (100 × v/v) with Spectra/por 3 membrane. The diffusate was lyophilized, dissolved in 0.1 M pyridine, and 1.5 ml of this sample were applied to a Dowex Ag-1-X8 200 to 400 mesh (Bio-Rad Laboratories, Richmond, Calif.) column (1.5 x 10.0 cm) equilibrated in 0.1 M pyridine, pH 8.6.

Elution with 0.1 M pyridine/0.5 M formic acid of −0.5 pH units/4 bed volumes was performed in the pH range 6.0 to 2.0. The eluate was pooled into 15 arbitrary fractions on the basis of pH. These fractions were lyophilized, resuspended in 7 ml of cold distilled water, and tested in duplicate for eosinophil chemotactic activity after dilution with RPMI 1640 medium (100 × v/v).

*Chemotactic activity of serum.* Peripheral blood of mice after 8 weeks of injection was collected by cutting the femoral artery under sterile conditions and allowed to clot at 22°C for 1 hr. Sera separated by centrifugation were pooled and stored at −70°C. The sera were tested for chemotactic activity either nondialyzed and diluted one, ten, or one hundred times with RPMI 1640 medium or dialyzed with Spectra/Por 3 membrane against 100 times volume of the same medium.

## RESULTS

*In vitro detection of chemotactic factor.* No eosinophil chemotactic activity was detected in both dialyzable and nondialyzable fractions up to the 4th week of infection. However, by the 5th week of infection the dialyzable fraction (m.w. <3500) in one of three mice showed a low degree of activity (Fig. 1). After 7 weeks of infection the activity increased significantly in all animals tested. In the same system there was no chemotaxis for monocytes, although Zymosan-activated guinea pig sera showed moderate chemotactic activity for monocytes. Polymorphonuclear chemotaxis was not examined. In addition, the nondialyzable residue had no chemotactic activity for eosinophils or monocytes. The experiment to test for reliability of eosinophil chemotaxis showed a reduction in number of migrated cells when 0.2 ml (average 27 ± 7 eosinophils/hpf) and 0.75 ml (average 14 ± 2 eosinophils/hpf) of dialyzate were added to both upper and lower compartments.

*Gel filtration with Sephadex G-25 chromatography.* A typical elution pattern of chemotactic activity detectable in liver extracts at 8 weeks after infection appears in Figure 2. The majority of eosinophil chemotactic activity was eluted in the fractions just after the molecular markers, vitamin $B_{12}$. Lyophilization did not affect the potency of activity.

*Effects of carboxypeptidase A, boiling, acidification, and alkalization.* Essentially no effect was observed of heat, acid, or alkaline treatment on chemotactic activity of the partially purified and pooled samples obtained from mice 8 weeks after infection (Table I). However, a marked reduction in activity occurred in carboxypeptidase-A treated samples. This result indicates that cleavage of peptide bonds caused the loss in the eosinophil chemotactic activity.

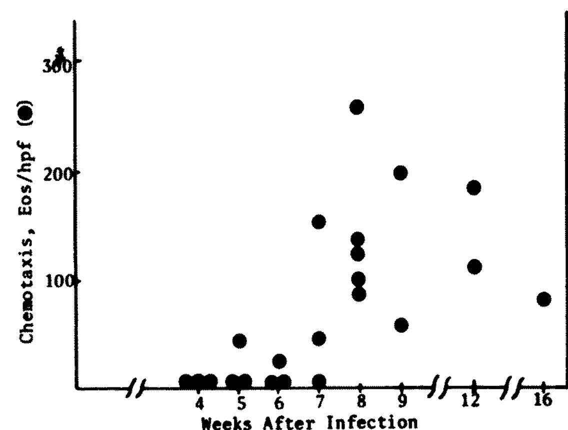


*Figure 1.* The time course response of eosinophil chemotactic factor derived from livers of mice infected with *S. mansoni.* Chemotactic activity (●) is expressed as an average of migrated cells per hpf in three filters for each animal. Zymosan-activated serum yielded an average of 308 ± 22 eosinophils/hpf. Spontaneous locomotion yielded an average of 0.7 ± 0.5 eosinophils/hpf.

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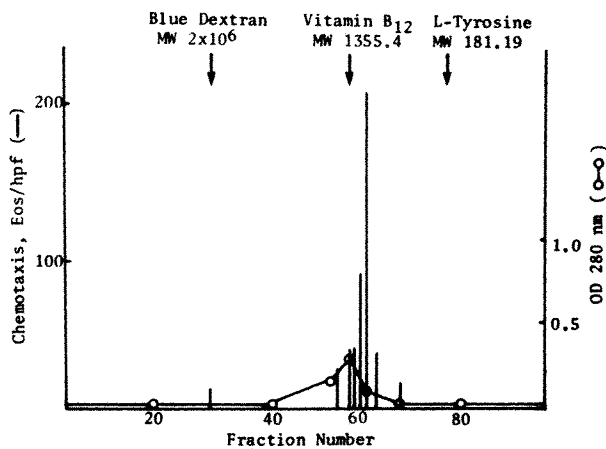


*Figure 2.* Sephadex G-25 gel filtration of eosinophil chemotactic factor in liver extracts of schistosome infected mice obtained 8 weeks after infection. Chemotactic response of eosinophils (——) and absorbance at 280 (○——○) are shown. Zymosan-activated serum yielded an average of 276 ± 5 eosinophils/hpf. No spontaneous locomotion of eosinophils was seen in this assay.

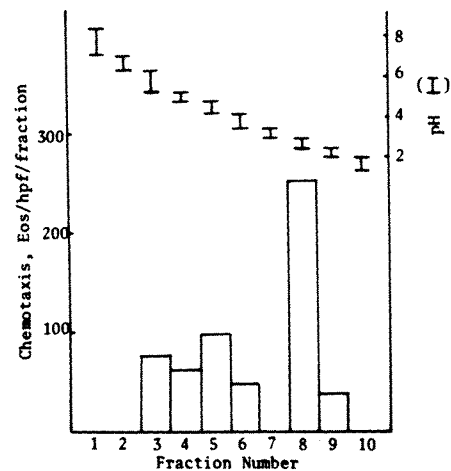


*Figure 3.* Dowex-1 anion exchange chromatography of fractions obtained from the major peak of eosinophil chemotactic activity of Sephadex G-25 gel filtration of schistosome liver extract. Chemotactic activity (*open bars*) is expressed as an average of migrated cells per hpf in three filters for each assay. Zymosan-activated serum yielded an average of 358 ± 8 eosinophils/hpf. Spontaneous locomotion yielded an average of 0.5 ± 0.3 eosinophils/hpf.

TABLE I

*Effects of boiling, acidification, alkalization and carboxypeptidase-A on eosinophil chemotaxis of ECF-G[a]*

| Materials Tested | Time | Eos/hpf ± S.E. |
|---|---|---|
| | *min* | |
| A. Starting samples | | 148 ± 27 |
| Simple boiling | 10 | 296 ± 28 |
| | 30 | 144 ± 6 |
| Boil in 1 N HCl | 10 | 191 ± 11 |
| | 30 | 186 ± 11 |
| Boil in 1 N NaOH | 10 | 211 ± 40 |
| | 30 | 199 ± 5 |
| B. Samples with carboxypeptidase-A | | 3 ± 2 |
| Samples without carboxypeptidase-A | | 119 ± 8 |

[a] The assay was quantified in triplicate and results were recorded as an average of the migrated eosinophils per hpf (Eos/hpf) in three filters for each sample. Zymosan-activated serum yielded an average of 337 ± 7 Eos/hpf for experiments (*A*), but 285 ± 9 for (*B*). Spontaneous locomotion of eosinophils yielded an average of 1.3 ± 0.7 Eos/hpf for experiments (*A*) but 1.9 ± 0.5 for (*B*).

*Dowex-1 anion exchange chromatography.* Eosinophil chemotactic activity eluted as a narrow peak at pH 3.1 to 2.6 and a broad band at pH 6.3 to 3.7 (Fig. 3). Further purification was not attempted, but it was noted that this partially purified material still had no chemotactic activity for monocytes.

*Chemotactic activity in serum.* No activity was demonstrated by the techniques used in the study.

## DISCUSSION

These results confirm the predicted appearance (9) of a low m.w. ECF-A-like peptide in experimental schistosomiasis. By G-25 gel chromatography, the peptide proved to have a m.w. of less than 1355 daltons and eluted approximately where ECF-A and similar eosinophil chemoattractants appear (10, 15–21). It was very strongly chemotactic for eosinophils, but not for mononuclear cells; polymorphonuclear cells were not tested. Chemotactic activity was virtually abolished by treatment with carboxypeptidase-A, but unlike ECF-A it was stable to boiling in both acid and alkali.

By Dowex-1 anion exchange chromatography, two fractions of chemotactic activity were separated. The less acidic broad fraction (pH 6.3 to 3.7) has some similarities to an intermediate m.w. eosinophil chemotactic material described by Boswell *et al.* (16), although its apparent m.w. is smaller. The other more acidic sharp peak of activity seems to correspond most closely to the less acidic minor tetrapeptide in tissue-extracted ECF-A and to a synthetic tetrapeptide Ala-Gly-Ser-Glu reported by Goetzl and Austen (17). Other similar low m.w. eosinophil chemotactic factors have been found in tumors associated with eosinophilia (18), the blister fluid from patients with pemphigoid (15), and from polymorphonuclear leukocytes under a number of circumstances (19–21). In addition, ECF-A-like activity has been extracted from basophils from several sources (22–24). Perhaps the chemotactic factor from schistosome-infected mice should be called ECF-G to distinguish it from others of this class at least until chemical structure is proven.

ECF-G differs in many ways from the lymphokine (ESP) found in schistosomiasis and characterized by Colley and colleagues (3, 4) and from the eosinophilopoietic substances isolated from spleens of *S. mansoni*-infected mice (8). Likewise, it does not appear to be related to the complement-dependent eosinophil chemotactic agent (ECF-C) (10). ECF-G also differs from histamine which shows eosinophil chemotactic activity *in vitro* (25, 26). It has a molecular size similar to a circulating eosinophilopoietic substance (eosinophilopoietin) generated by administration of anti-eosinophil serum to mice (27), but that material is heat-labile and shows no eosinophil attractant properties (27).

In cutaneous anaphylaxis two patterns of eosinophil chemotactic activity have been described. ECF-A appears early (28, 29) and a higher m.w. attractant accounts for delayed incursion of eosinophils (29). In schistosome-induced hepatic granulomas, no ECF-A-like activity was detected before the 5th week of infection. Appearance of ECF-G paralleled the time course of development of egg granulomas and the increase of mast cells in the granulomas (30). As for infiltration of eosinophils in the granulomas, they are seen earlier in the perivascular regions, and their major accumulation in the livers occurs with enlargement of the granulomas which consist of mononuclear cells and

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macrophages. The cell types in granulomatous livers responsible for production or secretion of ECF-G are unknown. We have also no direct evidence to eliminate the possibility that ECF-G is a product of eggs or worms. It is possible that mast cells may release the factors as in the case of ECF-A. However, another possibility is that mononuclear cells or macrophages may secrete this substance, since their secretory prowess remains to be divined (31). In addition, monocytes may act to stabilize chemoattracted eosinophils, since Kownatzki *et al.* (32) reported that monocytes release an eosinophil-immobilizing factor. An interaction of ECF-G and macrophages may account for the unique histopathology of granulomas seen in schistosomiasis.

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