[Jpn. J. Parasitol., Vol. 35, No. 2, 121–126, April, 1986]

Eosinophil Chemotactic Factor-Release from Guinea Pig Neutrophis after *In Vitro* Stimulation with *Strongyloides ratti* Larvae

MAKOTO OWHASHI[1], TATSUYA ABE[1], MASATAKA KORENAGA[2] AND YUKIFUMI NAWA[1]

(Received for publication, July 11, 1985)

Key words: *Strongylodes ratti*, third stage larvae (L3), guinea pig, eosinophil chemotactic factor (ECF), neutrophils

Introduction

An increase in number of eosinophils both in tissue and in the circulation is one of the common cellular responses of the host against helminth infection (Beeson and Bass, 1977). To account for such eosinophil responses, both parasite-derived and host-derived eosinophil chemotactic factors (ECFs) has been reported (Goetzl *et al.*, 1983). As host derived ECFs, mast cell-derived ECF-A, lymphocyte-derived ECF lymphokine, and complement-derived ECFs are well-known. In addition, recently König and coworkers (König *et al.*, 1976, 1978; Czarnetzki *et al.*, 1976) reported that neutrophils could generate and release ECF in response to phagocytosis or to other surface stimuli. As to the stimuli derived from parasites to cause ECF release from neutrophils, Czarnetzki (1978) reported ECF release from human netrophils by *Nippostrongylus brasiliensis* larvae. Furthermore, neutrophil stimulating factor, which could stimulate ECF release from guinea pig neutrophils, has been isolated and characterized from soluble egg antigen (SEA) of *Schistosoma japonicum* (Owhashi *et al.*, 1985). Thus, neutrophil-derived ECF seems to play an important role in the mediation of eosinophilia associated with parasite infections.

In the present study, we have examined ECF release from neutrophils after stimulation with *Strongyloides ratti* larvae and found that live larvae were the most effective stimuli in causing ECF release from netrophils.

Correspondece: Dr. M. Owhashi, Department of Parasitology, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, Japan.

[1]*Department of Parasitology, Miyazaki Medical College, Miyazaki 889-16, Japan.*

[2]*Department of Parasitic Diseases, Kumamoto University Medical School, Kumamoto 860, Japan.*

Materials and Methods

Preparation of Strongyloides ratti *larvae*

A strain of *S. ratti* was supplied by Prof. I. Tada (Department of Parasitic Diseases, Kumamoto University Medical School, Japan) and has been maintained in our laboratory by serial passage in Wistar rats. Pellets of faeces were collected 7–14 days after subcutaneous infection with 3,000 infective larvae and were cultured by the filter paper method. Larvae were extensively washed with sterile saline and resuspended in phosphate buffered saline (PBS) containing 100 U/ml Penicillin and 100 mg/ml Streptomycin. After incubation for 1 hr, larvae were washed with sterile PBS and finally resuspended in RPMI 1640 culture medium

(GIBCO) without serum. Heat treatment was performed at 56°C for 5 min. *S. ratti* larvae were completely killed by this treatment.

Excretory-secretory products (ES) from *S. ratti* larvae were prepared by culturing 10,000 larvae/ml in RPMI 1640 without serum at 37°C for 90 min in a humidified atmosphere with 5% CO_2. Culture supernatant was passed through Millipore filter (0.45 μm) and immediately served for experiments.

Preparation of netrophils and eosinophils

Peritoneal exudate rich in eosinophils was prepared according to the methods described previously (Owhashi and Ishii, 1982). Neutrophils were also collected as peritoneal exudate cells of guinea pigs. They received intra-peritoneal injection of 20 ml of 0.1% oyster glycogen (Nakarai Chemicals) 4 hr before use. The exudated cells were collected in 50 ml of PBS, washed twice with PBS, and resuspended in RPMI 1640 medium.

Assay of chemotaxis in vitro

Chemotactic activity for eosinophils was measured according to the method described previously (Owhashi and Ishii, 1982) with a slight modification in the procedure. In short, blind-well type chemotactic chambers (Bio-Rad Laboratories, Richmond, CA, USA) were equipped with Millipore membrane filters (Millipore Co., Bedford, Mass., USA) with a pore size of 3 μm. Eosinophil-rich cell suspensions from three or four animals were pooled. The cell number was adjusted to 1×10^6/ml with RPMI 1640 medium containing 10% heat-inactivated homologous serum. Incubation was performed at 37°C for 3 hr for eosinophil chemotaxis. The membranes were stained according to Litt's procedure (Litt, 1963). The number of migrated eosinophils was counted in 10 high power field (hpf) at 10 × 40 magnification by the same procedure described previously (Owhashi and Ishii, 1982; Owhashi *et al.*, 1985). The chemotactic activity was expressed as the mean and standard error of mean (S.E.M.) of 4 filters.

Assay for neutrophil stimulating activity

The activity of neutrophil stimulation to induce ECF release from neutrophils was assayed as follows: The larval suspension was added to the equal volume of neutrophil-rich cell suspension (more than 90% of neutrophils) in RPMI 1640 medium. The density of larvae and the cells were stated in each experiment. The mixture was incubated at 37°C with continuous shaking for 20 min except in the time course experiment. Then the mixture was centrifuged at 1000 rpm for 5 min. The supernatant was assayed for eosinophil chemotactic activity.

Results

Time course study of ECF release from neutrophils

Kinetics of ECF release from neutrophils after stimulation with live *S. ratti* larvae was examined. Neutrophils (99% neutrophils, 1% eosinophils, 3×10^6 cells/ml) were incubated with 10,000 larvae/ml at 37°C for 5, 10, 20, 40 and 60 min, and ECF activity in the super-

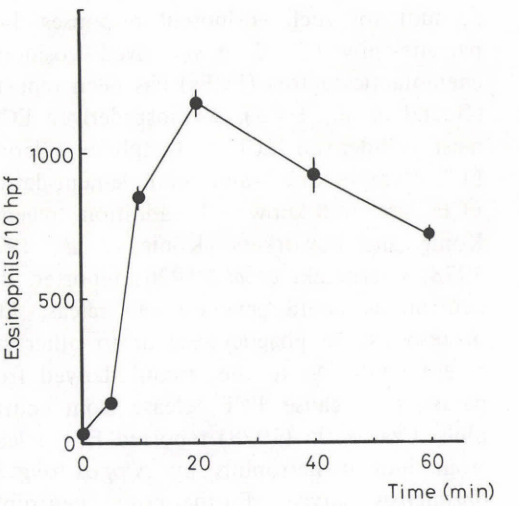

Fig. 1 Time course of ECF release from neutrophils after stimulation with *Strongyloides ratti* larvae.

Neutrophils (99% netrophils, 1% eosinophils, 3×10^6 cells/ml) were incubated with 10,000 *S. ratti* L3 at 37°C. The supernatant was examined for eosinophil chemotactic activity. Eoshinophil chemotactic activity was expressed as the mean ± S.E.M. (vertical line) of four filters.

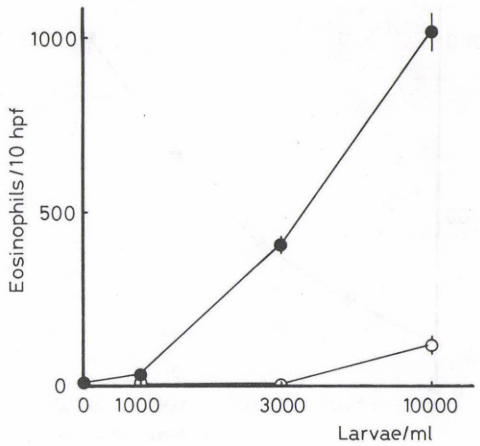

Fig. 2 Release of ECF from neutrophils after stimulation with various numbers of *Strongyloides ratti* larvae.

Various numbers of *S. ratti* L3 were incubated with (●——●) or without (○——○) neutrophils (3 x 10^6 cells/ml). Eosinophil chemotactic activity was expressed as the mean ± S.E.M. Incubation was performed at 37°C for 20 min, and the supernatant was examined for eosinophil chemotaxis.

natant was examined (Fig. 1). ECF release from neutrophils was observed as early as 5 min of incubation and reached a peak at 20 min of incubation. At 60 min of incubation, significant reduction of the eosinophil chemotactic activity was observed.

Effects of the dose of S. ratti *larvae on ECF release*

When 3 x 10^6 neutrophils were incubated with varying numbers of live *S. ratti* larvae at 37°C for 20 min, the release of ECF was proportional to the numbers of larvae added in the culture (Fig. 2). As a control, the same numbers of *S. ratti* larvae alone were cultured without neutrophils. As shown in Fig. 2, only weak ECF activity was detected in the culture supernatant of 10,000 larvae/ml.

Effects of the dose of cells on ECF release

When varying numbers of neutrophils were incubated with 10,000 *S. ratti* larvae, the eosinophil chemotactic activity in the supernatant was proportional to the number of cells up to 3 x 10^6 cells/ml. Slight ECF activity was observed in the culture supernatant from *S. ratti* larvae alone.

Table 1 Neutrophil stimulating activity of live or heart-killed *Strongyloides ratti* L3 measured by ECF release

Treatments	Activity (Eos./10 hpf ± S.E.)
heat 56°C 5 min	0
control	323 ± 25

3 x 10^6 neutrophils were incubated with either 10,000 live or heat-killed *Strongyloides ratti* L3 for 20 min at 37°C. ECF activity in the supernatant was measured.

Table 2 Neutrophils stimulating activity of the excretory-secretory products (ES) of *Strongyloides ratti* L3 measured by ECF release

Neutrophils	ES	Activity (Eos./20 hpf ± S.E.)
+	+	38 ± 7
–	+	0
+	–	6 ± 2

ES was prepared by incubating 10,000 L3/ml for 90 min. After Millipore filtration, an equal amount of ES and 6 x 10^6/ml neutrophils were mixed and cultured for 20 min. Fresh medium was added to control cultures of neutrophil alone or ES alone.

Effect of heat-killed S. ratti *larvae on ECF release*

To examine whether ECF release from neutrophils is related to the viability of *S. ratti* larvae, 10,000 heat-killed (56°C, 5 min) larvae were added to 3 x 10^6/ml of neutrophils. As shown in Table 1, only live *S. ratti* larvae were able to stimulate neutrophils to release ECF.

Effect of ES on ECF release

Since previous experiment showed that the viability of *S. ratti* larvae is essential to stimulate neutrophils, the possibility that ES released from *S. ratti* larvae could stimulate neutrophils to release ECF was examined. For this purpose, *S. ratti* larvae were cultured at a concentration of 10,000 larvae/ml and the culture supernatant was harvested 90 min after incubation. Millipore-filtered culture supernatant was added to the equal volume of 3 x 10^6/ml neutrophils. As shown in Table 2, weak but significant ECF release was observed by culturing neutrophils with ES.

Discussion

The results reported here show that live *S. ratti* larvae are able to stimulate neutrophils to cause the release of ECF. Concerning neutrophil-derived ECF, ECF release after stimulation with calcium ionophore A23187 (Czarnetzki *et al.*, 1976), archidonic acid (König *et al.*, 1978), Phospholipase A (Tesch and König, 1980), melitin (Kroegel *et al.*, 1981), and during phagocytosis of zymosan (König *et al.*, 1976) has been reported. Our results, together with Czarnetzki (1978)'s earlier study of ECF release from human neutrophils after stimulation with *Nippo-strongylus brasiliensis* larvae, indicate that at least infective larvae of some helminth species are able to stimulate ECF release from neutrophils.

In terms of the mechanism of ECF release from neutrophils, heat-killed larvae were completely ineffective in causing ECF release from neutrophils. Although Czarnetzki (1978) reported that frozen immotile larvae were, after opsonization, just as effective as fresh larvae, our result rather indicate that the viability of larvae is critical under the absence of antibody and/or complement. Since live *S. ratti* larvae were highly effective in causing ECF release from neutrophils and ES from *S. ratti* larvae have weak but significant stimulating activity, not only phagocytosis of parasite products or membrane stimulation by ES but also mechanical agitation of the cells by active movement of larvae may be a powerful signal for neutrophils to release ECF.

Related to this, Goto and Kitamura (1984) reported that the production of lymphocyte stimulating factor by human peripheral blood neutrophils was augmented by mechanical shaking of the cells.

In the present study the decline of the chemotactic activity of the released ECF after prolonged stimulation was observed (Fig. 1). Similar phenomenon has been reported by Czarnetzki *et al.* (1976), and has been explained by the existence of inactivator released together with ECF from neutrophils

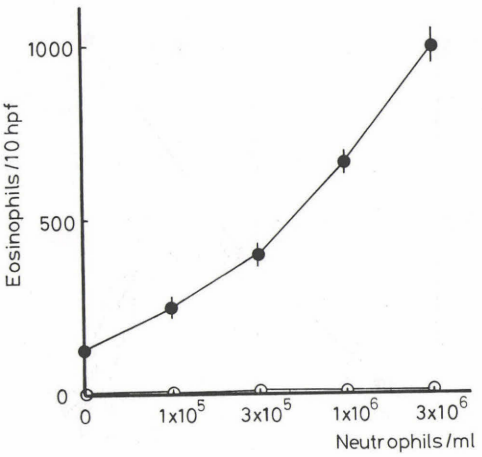

Fig. 3 Release of ECF from neutrophils at different cell concentrations.

Various concentrations of neutrophils were incubated with (●——●) or without (○——○) 10,000 *S. ratti* L3. Eosinophil chemotactic activity was expressed as the mean ± S.E.M. Incubation was performed at 37°C for 20 min, and the supernatant was examined for eosinophil chemotaxis.

(Czarnetzki *et al.*, 1976; Frickhofen and König, 1979).

In conclusion, demonstraiton of neutrophil stimulating activity of *S. ratti* larvae to release ECF suggested a possible role of neutrophils for one of important sources of ECF in the eosinophil accumulation (Dawkins *et al.*, 1981) around tissue migrating *S. ratti* larvae.

Summary

When guinea pig neutrophils were cultured with live *Strongyloides ratti* larvae, eosinophil chemotactic factor (ECF) was released in the culture supernatant. The release of ECF from neutrophils began as early as at 5 min and reached a peak at 20 min. ECF release was dependent on both the number of larvae and the number of cells in the culture. Heat-killed *S. ratti* larvae at 56°C for 5 min was completely ineffective in causing ECF release form neutrophils. On the other hand, excretory-secretory products (ES) of *S. ratti* larvae had

significant neutrophil stimulating activity to cause the release of ECF. From these results, possible mechanism of ECF release from neutrophils after stimulation with live parasite larvae was discussed.

Acknowledgments

The authors are grateful to Professor I. Tada for allowing us to perform collaborative work, and also wish to thank Dr. Y. Horii for the constructive critisism.

References

1) Beeson, P. B. and Bass, D. A. (1977): The Eosinophil, Volume XIV in the series of Major Problems in Internal Medicine, ed. by Lloyd H. Smith, Jr., Saunders, Philadelphia, 161–189.
2) Czarnetzki, B. M. (1978): Eosinophil chemotactic factor release from neutrophils by *Nippostrongylus brasiliensis* larvae. Nature, 271, 553–554.
3) Czarnetzki, B. M., König, W. and Lichtenstein, L. M. (1976): Eosinophil chemotactic factor (ECF). I. Release from polymorphonuclear leukocytes by the calcium ionophore A23187. J. Immunol., 117, 229–234.
4) Dawkins, H. J. S., Muir, G. M. and Grove, D. I. (1981): Histopathological appearances in primary and secondary infections with *Strongyloides ratti* in mice. Int. J. Parasitol., 11, 97–103.
5) Frickhofen, N. and König, W. (1979): Subcellular localization of the eosinophil chemotactic factor (ECF) and its inactivator in human polymorphonuclear leucocytes (PMN). Immunology, 37, 111–122.
6) Goetzl, E. J., Foster, D. W. and Golden, D. W. (1983): Receptor-directed modulation of human eosinophil function. In Immunobiology of the Eosinophil, ed. by T. Yoshida and M. Torisu, Elsevier, New York, 61–76.
7) Goto, F. and Kitamura, K. (1984): Interleukine 1 class molecule: produciton, physicochemical nature and its biological significance. Minophagen Med. Rev., 29, 47–66 (in Japanese).
8) König, W., Czarnetzki, B. M. and Lichtenstein, L. M. (1976): Eosinophil chemotactic factor (ECF). II. Release from polymorpphonuclear leukocytes during phagocytosis. J. Immunol., 117, 235–241.
9) König, W., Tesch, H. and Frickhofen, N. (1978): Generation and release of eosinophil chemotactic factor from human polymorphonuclear neutrophils by arachidonic acid. Eur. J. Immunol., 8, 434–437.
10) Kroegel, C., Konig, W., Mollay, C. and Kreil, G. (1981): Generation of the eosinophil chemotactic factor (ECF) from various cell types by melittin. Mol. Immunol., 18, 227–236.
11) Litt, M. (1963): Studies in experimental eosinophilia. V. Eosinophils in lymph nodes of guinea pigs following primary antigenic stimulation. Am. J. Pathol., 42, 529–549.
12) Owhashi, M. and Ishii, A. (1982): Purification and characterization of a high molecular weight eosinophil chemotactic factor from *Schistosoma japonicum* eggs. J. Immunol., 129, 2226–2231.
13) Owhashi, M., Horii, Y. and Ishii, A. (1985): Isolation of *Schistosoma japonicum* egg-derived neutrophil stimulating factor: its role on eosinophil chemotactic factor release from neutrophils. Int. Archs Allergy appl. Immunol. 78, 415–420.
14) Tesch, H. and König, W. (1980): Phospholipase A and arachidonic acid: a common link in the generation of the eosinophil chemotactic factor (ECF) from human PMN by various stimuli. Scand. J. Immunol., 11, 409–418.

Strongyloides ratti 幼虫刺激によるモルモット好中球からの好酸球遊走因子の放出

大橋　真[1)]・阿部達也[1)]・是永正敬[2)]・名和行文[1)]
([1)] 宮崎医科大学寄生虫学教室，[2)] 熊本大学医学部寄生虫病学教室)

Strongyloides ratti 幼虫とモルモット好中球を混合培養することにより，好中球からの好酸球遊走因子 (ECF) の放出が見られた．ECFの放出は培養開始後わずか5分でも起こり，20分でピークに達した．ECFの放出は，混合培養する幼虫数及び好中球数に比例して増加した．56℃，5分の熱処理により殺した *S.ratti* 幼虫では，好中球からのECFの放出をおこす活性は見られなかった．一方，*S. ratti* の分泌産物 (ES) でも弱いながら好中球を刺激してECFの放出をおこすことが可能であった．これらの結果から，幼虫刺激による好中球からのECF放出の機構について考察した．