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Direct Attraction of Neutrophils *in vivo* by the Purified Neutrophil Chemotactic Factor of *Dirofilaria immitis* (NCF-Di) and Its Secretion from Adult Worms*

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Abstract: The neutrophil chemotactic factor (NCF-Di) has recently been purified from *Dirofilaria immitis* as a polypeptide by us. In the present study, *in vivo* activity of NCF-Di and its secretion from the adult worm of *D. immitis* were investigated. When various concentrations of NCF-Di (0.5 to 500 μg/ml) were injected intradermally into the back of guinea pigs, a large number of neutrophils infiltrated at the injection sites 2 hours after the injection. The severity of the reaction reached the peak at 6 hours and was dose dependent. Whereas only a few other cells infiltrated. It is indicated that the neutrophil chemotactic factor of *D. immitis* could directly and selectively attract neutrophils *in vivo*. When intact adult worms of *D. immitis* were cultured, significant neutrophil chemotactic activity was detected in the culture supernatant of worms. This result suggests possible secretion of the neutrophil chemotactic factor by *D. immitis* adults in the hosts.

Key words: *Dirofilaria immitis*, Neutrophil, Neutrophil chemotactic factor (NCF)

The neutrophil chemotactic factor (NCF-Di) has recently been purified from *Dirofilaria immitis* adult worm extract. It is a polypeptide having a molecular weight of 14,000-17,000, and its physicochemical properties have also been characterized (Horii *et al.*, 1986). The present study was designed to examine *in vivo* activity of NCF-Di and to investigate whether NCF was released from the worms of *D. immitis* or not.

The purified NCF of *D. immitis* was prepared according to the previous report (Horii *et al.*, 1986). Protein concentration was adjusted by the method of Lowry *et al.* (1951).

Live *D. immitis* adult worms were collected from heart of dogs at necropsy under sterile condition. Worms were washed with sterilized phosphate-buffered saline (PBS, pH

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7.4), then used in the experiment immediately. Ten male and five female worms were cultured separately in glass dishes with 5ml of PBS plus antibiotics (150 IU penicillin and 150 μg streptomycin/ml) at 37℃. At intervals (0, 0 to 30, 30 to 90, 90 to 150, 150 to 210 and 210 to 270 minutes), culture fluids were collected and 5ml of fresh medium added. The culture fluids were centrifuged at 3,500 rpm for 10 minutes. Neutrophil chemotactic activity of the supernatant was tested by the *in vitro* chemotaxis assay.

In vivo chemotaxis was carried out using Hartley strain guinea pigs as previously (Horii *et al.*, 1984). Animals were injected intradermally into the back with 0.1ml of sterilized NCF-Di (0.5, 5, 50, and 500 μg/ml). Ovalbumin (50 μg/ml) and PBS were usd as negative controls. Groups of three animals were killed at 2, 6 and 24 hours after the injection and the tissue of injection sites were collected immediately. The specimens were fixed in 10% formalin solution, embedded in paraffin, sectioned at 4μm and stained with hematoxylin eosin. A total of 5 randomly selected high power fields (hpf) at 10×40 magnification for each section were examined in the upper limit of the panniculus carnosus and the reticular layer of dermis for neutrophils, and the total number of migrating cells was counted. The data reported here was based on the mean and standard error of mean (SEM) of cell numbers obtained from three animals in each group.

In vitro chemotaxis was carried out using Blind-well chambers with Millipore filters having a pore size of 3μm and guinea pig neutrophils (2×10^6/ml) as the indicator cells according to the previous report (Horii *et al.*, 1986). After 2 hours incubation of the chambers at 37℃ in a 5% CO_2 atmosphere, membranes were stained according to the method of Litt (1963). The migrated neutrophils were counted as the method described previously (Horii *et al.*, 1986). The total number of migrated neutrophils at 10 hpf was counted. Chemotactic activity was expressed as the mean±SEM of the total numbers from 4 filters.

As shown in Fig. 1, a large number of neutrophils infiltrated at the injection sites of NCF-Di 2 hours after the injection even at low concentration of protein (0.5 μg/ml). The cell numbers were increased and reached their peaks at 6 hours in dose dependent manner. Whereas either no eosinophils or only a few other cells were observed at the injection site.

As shown in Fig. 2, high neutrophil chemotactic activity was detected only in the culture supernatant of female worms during the early period of incubation (0 to 90 minutes).

The present data clearly indicates that the NCF of *D. immitis* attracts neutrophils directly in the tissues of normal animals. Moreover, this NCF was released from adult worms. These facts seem to be important to consider possible roles of NCF in the infection of filariae. Recently, Shigeno *et al.* (1987) reported that neutrophil infiltration was observed around the inoculated *Brugia pahangi* larva in the hamster tissue. If *B. pahangi* larvae have similar factor, it might cause such neutrophil infiltrations. Although neutrophils have been identified as one of the effector cells against parasites (Butterworth, 1984), roles of neutrophils in the real inflammatory lesions caused by parasites are complicated. It has

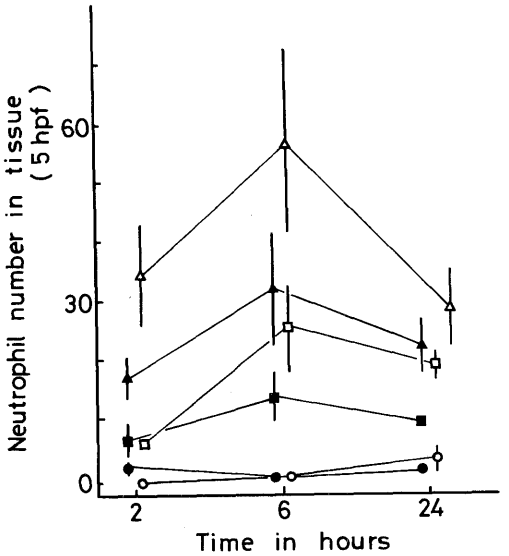

Fig. 1. Neutrophil infiltration in the tissues after the intradermal injection of the purified neutrophil chemotactic factor (NCF; ■=0.5, □=5, ▲=50, and △=500 μg/ml), ○=ovalbumin (50 μg/ml), and ●=PBS in normal Hartley strain guinea pigs. In each section a total of 5 hpf (×400) were counted for neutrophils. The data is based on the mean ± SEM of cell numbers obtained from three animals.

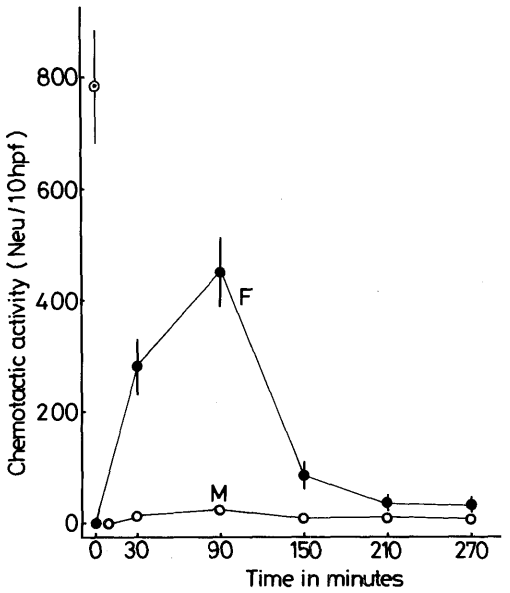

Fig. 2. Time-course of neutrophil chemotactic factor release in culture medium from 10 male (M) and 5 female (F) adult worms of *D. immitis*. Chemotactic activity was measured with guinea pig neutrophils as indicator cells. This activity is expressed as the total count of migrated cells in the 10 hpf of the Millipore filter. The data is based on the mean ± SEM of cell numbers in 4 filters. ◎=positive control (soluble egg extract of *Schistosoma japonicum*, 500 μg/ml).

been reported that neutrophils could not damage the larva of *Oesophagostomum aculeatum* in the inflammatory lesion in monkeys, in contrast to complete destruction of worms by eosinophils (Horii *et al.*, 1985). This report suggests that neutrophils do not always play an effector role in the inflammatory site. Since we have not a good laboratory model for investigation of immunological event in *D. immitis* infection, real role of the NCF of *D. immitis* in the infection still remain unclear. More recently, neutrophil chemotactic activity was detected in other species of filariae which can be maintained in laboratory animals (unpublished data). Therefore, roles of such NCF in the real infection should be further clarified using laboratory models.

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犬糸状虫の好中球遊走物質 (NCF-Di) の *in vivo* での作用と虫体からの放出

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最近，我々は犬糸状虫 (*Dirofilaria immitis*) より好中球遊走活性を持つポリペプチド (NCF-Di) を単離・精製した．本研究では NCF-Di の *in vivo* での作用と，虫体からの放出の有無について調べた．NCF-Di を 0.5, 5, 50, 500 μg/ml の濃度でモルモット皮内に注射すると，2時間後には注射部位に著明な好中球の浸潤がみられた．その反応は注射後6時間をピークとするもので、浸潤細胞数は濃度に依存して増加した．また，注射部位には好中球以外の細胞はわずかしかみられなかった．この事から，犬糸状虫由来の NCF-Di はモルモット体内で好中球を直接かつ選択的に誘引する事が明らかとなった．また，犬糸状虫成虫を短時間培養すると培養液中に著明な好中球遊走活性が認められることから，この物質 (NCF-Di) は虫体から放出 (排泄) されているものである事がわかった．

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