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ORIGINAL PAPER

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Kinetics of change in the eotaxin concentration in serum and cerebrospinal fluid of mice infected with *Angiostrongylus cantonensis*

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Abstract The kinetics of changes in the eotaxin concentration in the serum and cerebrospinal fluid (CSF) of BALB/c mice after infection with *Angiostrongylus cantonensis* and the correlation between the concentration of eotaxin and worm recovery were investigated. The mean concentration of eotaxin in serum of infected mice gradually increased from 46.3 ± 6.5 pg/ml at week 0 to 104.9 ± 44.8 pg/ml at week 3 after infection, while the mean eotaxin level in the CSF of infected mice rapidly increased from 18.7 ± 2.1 pg/ml to 193.2 ± 23.6 pg/ml 1 week after infection and then increased further to 507.8 ± 167.9 pg/ml at week 3. The concentrations of eotaxin in the CSF of infected mice each week after infection were all significantly higher than those in serum ($P < 0.0001$). In parallel with the increase in eotaxin in the CSF, infected mice showed gradual increases in CSF eosinophilia and a reduction in intracranial worm recovery. The concentration of eotaxin in CSF was higher in infected mice with more worms in the brain, except when the number of worms in the brain was > 30. In addition, when the worm counts in the brains of infected mice were < 30, eotaxin concentrations in the CSF were positively correlated with worm counts in the brain ($P < 0.001$). Thus, the release of eotaxin in the CSF of mice infected with *A. cantonensis* observed in this study was time dependent and worm-load dependent, and in parallel with the increase in eotaxin in the CSF, and gradual decreases in worm counts in the brains of infected mice.

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Introduction

Angiostrongylus cantonensis is a rat lungworm. When non-permissive hosts, e.g. mice, guinea pigs, rabbits and humans, ingest the third-stage larvae (L3) of this parasite, the larvae migrate to the brain and spinal cord where they develop into fifth-stage larvae (L5) and then die. *A. cantonensis* is the causative pathogen of human eosinophilic meningitis and meningoencephalitis, which is distributed in the South Pacific islands and Southeast Asia (Alicata 1965).

As an animal model for humans, mice are infected with *A. cantonensis* for research. During infection with *A. cantonensis*, mice especially evoke marked increases in eosinophils in the cerebrospinal fluid (CSF), reaching peak levels at around day 20. In parallel with this CSF eosinophilia, infected mice show gradual reductions in intracranial L5 recovery (Sugaya and Yoshimura 1988). In both cases of patients and experimental mice infected with *A. cantonensis*, the percentage of eosinophils among leukocytes in the CSF is significantly higher than that in the peripheral blood (Punyagupta et al. 1975; Sugaya and Yoshimura 1988; Hwang and Chen 1991; Carlisle et al. 1998; Yoshimura et al. 2000).

It is interesting to note which components in the brains of infected patients and other non-permissive hosts recruit eosinophils from the peripheral circulation to the brain. Eotaxin is a potent and selective chemoattractant for eosinophils during inflammation and allergic reactions (Rothenberg et al. 1995). Eotaxin, a member of the CC chemokine family of inflammatory and immunoregulatory cytokines, was first described in the bronchoalveolar lavage fluid of allergen-challenged guinea pigs (Jose et al. 1994a, 1994b). Eotaxin couples with CC chemokine receptor 3 expressed on eosinophils, causing selective recruitment of eosinophils to the sites of inflammation (Kitaura et al. 1996; Daugherty et al. 1996; Ponath et al. 1996a). CC chemokine receptor 3 mRNA and its expressed proteins are significantly elevated in the bronchial mucosa and skin of patients

with asthma and atopic dermatitis (Ying et al. 1997; Yawalkar et al. 1999). Eotaxin is the most extensively studied chemoattractant in different animal models of asthma (Teran 2000), but little is known about its role in parasitic helminth infections. Currently, no information on the possible involvement of eotaxin in *A. cantonensis* infections is available. Thus, the kinetics of eotaxin change in serum and CSF of mice after infection with *A. cantonensis* and the correlation between the concentration of eotaxin and worm recovery in mice was investigated in this study.

Materials and methods

Parasite and infection

The parasite *A. cantonensis* used in the present study was originally obtained from a field mollusc host and has been propagated for several years in our laboratory by cycling it through rats and snails (*Biomphalaria glabrata*). *A. cantonensis* L3 were harvested from *B. glabrata* snails after treatment with artificial gastric juice.

Male BALB/c mice aged between 6 and 8 weeks, purchased from the National Laboratory Animal Breeding Research Centre, were raised and maintained in our laboratory animal centre. Mice were housed in an animal room with controlled light (12 h illumination), temperature (22 ± 2°C) and humidity (50 ± 10% relative humidity). Twenty-four mice were orally infected with 50 *A. cantonensis* L3 via a tube inserted into the stomach after slight ether anesthesia. Eight mice were sacrificed every week after infection. We divided another 24 mice into three equal groups. The mice in each group were infected with 40, 30 and 15 L3, respectively, and all of them were sacrificed at day 21 after infection.

Collection of specimens

Blood samples were collected by a cardiac puncture of experimental mice under deep ether anesthesia, and serum samples were stored at −20°C after centrifugation until further use. The skull was opened after mice were bled as completely as possible. The brain was removed and placed into a Petri dish and washed with 150 μl of minimum essential medium (MEM). In the meantime, the cerebral ventricles and cranial cavity were washed with 150 μl MEM. CSF was, thus, harvested with MEM from the above, which was then centrifuged in a small plastic centrifuge tube at 260× *g* for 10 min at 4°C. The supernatant was stored at −20°C until further use and the resultant sediments were then resuspended in 30 μl MEM for preparing smears for differential cell counts.

Eosinophil counts

For the determination of eosinophils, 10 μl of CSF rich in white blood cells concentrated from the cerebral ventricles and cranial cavity in the washed medium were smeared on a glass slide. All smears were stained with May-Grunwald/Giemsa solution. Briefly, the methanol-fixed cells were immersed in Giemsa buffer for 5 min and then stained with May-Grunwald solution (Muto Pure Chemicals, Japan) for 5 min. After the slides were rinsed in Giemsa buffer and blotted dry, the Giemsa solution (Muto Pure Chemicals) was added for 15 min. The slides were appropriately destained in the Giemsa buffer and then were examined for cells under a microscope after the slides had been air dried. Differential counts, of eosinophils in particular, were made on the basis of 500 nucleated cells in CSF.

Eotaxin measurement

Concentrations of eotaxin in serum and CSF samples were assayed according to the procedures of a commercial eotaxin assay kit (R & D Systems, USA). Briefly, eotaxin in serum and CSF specimens bound to antibodies specific for eotaxin that were coated in the wells of a microplate in the incubation procedure. After unbound components were removed by washing the wells, horseradish peroxidase conjugated with antibody against mouse eotaxin was added to each well. The wells were washed after 1 h of incubation and this was followed by the addition of substrate solution for the development of colour. The optical densities were read at 450 nm with a colorimeter (MR 5000; Dynatech, Billinghurst, UK) after a stop solution was added to each well.

Worm recovery

The brains of infected mice were shredded into small pieces. The worms were picked out and counted under a dissecting microscope.

Statistics

Results are presented as means ± SDs. Differences between groups were tested for significance using Student's *t* -test. The relationships between worm recovery and eotaxin concentrations were tested using the *F* -test. A *P* -value < 0.05 was considered significant.

Results

Eotaxin kinetics in serum and CSF of infected mice

The kinetics of eotaxin concentration change in serum and CSF of BALB/c mice infected with 50 L3 of *A. cantonensis* are shown in Table 1. The mean eotaxin concentration in serum of infected mice gradually increased from 46.3 pg/ml at week 0 to 104.9 pg/ml at week 3 after infection. The increase in eotaxin in serum became significant in the first week after infection compared to that before infection (Student's *t* -test, $P < 0.05$). The mean eotaxin level in CSF of infected mice rapidly increased from 18.7 pg/ml to 193.2 pg/ml at week 1, and then increased to 507.8 pg/ml at week 3 after infection. The concentrations of eotaxin in CSF of infected mice each week after infection were all signifi-

Table 1 Kinetics of changes in the eotaxin concentration (pg/ml) in serum and cerebrospinal fluid (*CSF*) of BALB/c mice infected with 50 third-stage larvae of *Angiostrongylus cantonensis*

Weeks after infection	No. of mice	Eotaxin levels (mean ± SD)	
		Serum	CSF
0	8	46.3 ± 6.5	18.7 ± 2.1
1	8	57.0 ± 8.9*	193.2 ± 23.6****
2	8	83.5 ± 17.6**	327.3 ± 56.9****
3	8	104.9 ± 44.8**	507.8 ± 167.9****

* $P < 0.05$
** $P < 0.01$
**** $P < 0.0001$ (eotaxin level in serum and CSF of infected mice compared to that in uninfected mice by Student's *t* -test)
**** $P < 0.0001$ (eotaxin level in CSF of mice after infection compared to that in serum by Student's *t* -test)

cantly higher than those in serum (Student's t -test, $P < 0.0001$).

Eosinophil counts

Eosinophil counts in CSF of BALB/c mice infected with *A. cantonensis* increased gradually at week 1 ($7\times10^3 \pm 316/\mu l$) and week 2 ($9\times10^3 \pm 289/\mu l$), then markedly increased at week 3 ($24\times10^3 \pm 513/\mu l$) after infection (Fig. 1). Most eosinophils assembled in the CSF of infected mice at week 3 after infection. The increases in eosinophils in CSF paralleled the kinetics of the change in eotaxin levels in the CSF of infected mice. Furthermore, in parallel with this CSF eosinophilia, infected mice showed gradual reductions in intracranial worm counts.

Eotaxin concentration at different infection intensity

The concentrations of eotaxin in serum and CSF of BALB/c mice infected with *A. cantonensis* at week 3 after infection with different amounts of worm (varying infection intensities) are shown in Table 2. There was a tendency toward higher CSF eotaxin production with higher intensities of infection. The highest eotaxin concentrations (130.2 ± 93.3 pg/ml in serum and 613.3 ± 177.3 pg/ml in CSF) were observed in mice in which the mean worm recovery in the brain was 24. However, the eotaxin concentration in the serum and CSF of mice with a mean worm recovery of 33 was lower than in mice with a mean worm recovery of 24. The increases in eotaxin in serum and CSF of mice at the four different infection intensities were all significantly higher than in those of uninfected mice (all $P < 0.05$ by Student's t -test). The concentrations of eotaxin in CSF samples were significantly higher than those in sera of mice at the four different infection intensities (all $P < 0.01$ by Student's t -test).

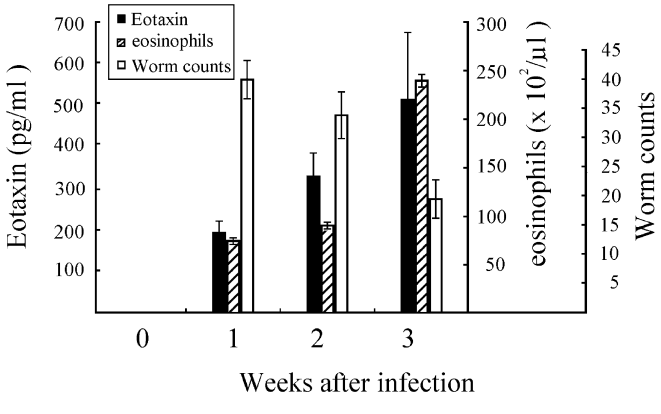

Fig. 1 Kinetics of eosinophil counts and eotaxin concentration changes in the cerebrospinal fluid (CSF) and worm counts in the brain of BABL/c mice infected with 50 L3 *Angiostrongylus cantonensis*. The increases in eosinophils in the CSF parallel the kinetic changes of eotaxin concentrations in the CSF of infected mice. In parallel with CSF eosinophilia, infected mice showed gradual reductions in intracranial worm counts, especially at week 3 after infection

Table 2 Mean ± SD of eotaxin levels in the serum and CSF among 40 BALB/c mice with different infection intensities at week 3 after infection

Infection intensity (worm count)	No. of mice	Concentration of eotaxin (pg/ml)	
		Serum	CSF
33 ± 2	8	119.2 ± 44.5	540.8 ± 223.2***
24 ± 2	8	130.2 ± 93.3	613.3 ± 177.3***
16 ± 3	8	82.0 ± 30.9	406.8 ± 106.9***
6 ± 2	8	57.2 ± 10.2	257.6 ± 81.5**
Uninfected	8	46.3 ± 6.5	18.7 ± 2.1

** $P < 0.01$
*** $P < 0.001$ (eotaxin concentration in CSF compared to that in serum by Student's t -test)

Correlation between eotaxin concentration and worm recovery

Figure 2 shows a scatter diagram illustrating the relationship between worm recovery and eotaxin concentrations in the CSF from 24 mice with worm burdens < 30 at week 3 after infection. Eotaxin concentrations in the CSF significantly correlated with worm counts in the brains of infected mice (F-test, $P < 0.01$).

Discussion

There is evidence showing that eosinophils play an important role in non-permissive host reactions against *A. cantonensis* after infection. For example, in an in vitro study, it was shown that rat eosinophils adhered to and killed young adult worms by immunoglobulin (Ig) G-dependent cell-mediated cytotoxic mechanisms (Yoshimura et al. 1983), and guinea pig eosinophils similarly adhered to L3 by IgE-dependent mechanisms and damaged them (Perez et al. 1989). Furthermore, in

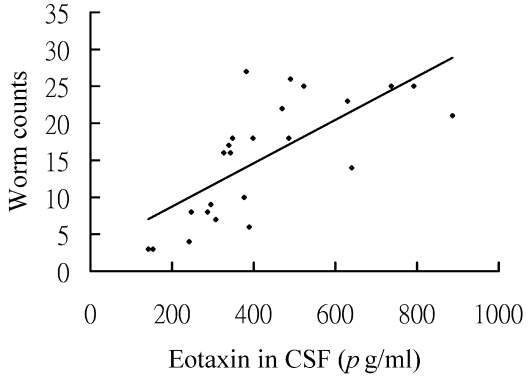

Fig. 2 Correlation between worm counts and eotaxin concentrations in the CSF of 24 infected BALB/c mice at week 3 after infection. The eotaxin concentration was positively correlated with worm count ($r = 0.71$, $P < 0.001$)

an in vivo study, when eosinophils gradually increased in the CSF of mice infected with *A. cantonensis*, the mice showed gradual reductions in intracranial worm recovery (Sugaya and Yoshimura 1988). *A. cantonensis* infection in IL-5-transgenic mice also demonstrated lower intracranial worm recovery and smaller female worms than in naive C3H/HeN mice (Sugaya et al. 1997).

Indeed, systemic and local eosinophilia occurring in mice and other non-permissive hosts infected with *A. cantonensis* are well known (Carlisle et al. 1998; Yoshimura et al. 2000; Yii 1976; Tsai et al. 2001). Punyagupta et al. (1975) reported that 70% of patients with eosinophilic meningitis or meningoencephalitis had blood eosinophil counts of about 10%, while Hwang and Chen (1991) reported that the percentage was 84% among their patients. Eosinophil counts in CSF were $>50\%$ in as many as 90% (Punyagupta et al. 1975) and 62.2% (Hwang and Chen 1991) of patients. Additionally, eosinophil counts in the CSF of three strains of mice at day 20 were significantly higher than those in peripheral blood after infection with *A. cantonensis* (Yoshimura et al. 2000).

Eosinophils differentiate in the bone marrow and mature in the blood. Nonetheless, most of them gradually migrate to the brain and spinal cord in non-permissive hosts infected with *A. cantonensis*. Eosinophil migration in response to chemokines is chemotactic, defined as the directed migration of cells toward an increasing gradient of chemoattractant molecules. Eotaxin is the strongest mediator involved in eosinophil chemotaxis and chemokinesis, which selectively recruits eosinophils (Simson and Foster 2000). In our study, all eotaxin concentrations assayed weekly in the CSF of the infected mice were significantly higher than those in the serum. Furthermore, the peripheral blood eotaxin concentration rapidly increased in week 1 and reached a maximum in week 3 after infection, and increased in parallel with the eotaxin concentrations in the CSF; infected mice showed gradual increases of eosinophils in the CSF. Therefore, the high concentration of eotaxin in the CSF is a very important reason for the migration and recruitment of eosinophils from the peripheral blood to the CSF in mice infected with *A. cantonensis*.

Constitutive eotaxin mRNA expression has been detected in multiple tissues and several kinds of cells (Rothenberg et al. 1995; Ganzalo et al. 1996; Ponath et al. 1996b; Quackenbush et al. 1997). Expression of eotaxin and eosinophilia are induced by Th2-associated immune responses (Li et al. 1998). An increase in eotaxin and then chemoattraction of eosinophils to surround parasites for host defence against them are observed in humans and experimental animals infected with some helminthic parasites (Evans et al. 1998; Ruth et al. 1998; Del Pozo et al. 1999). However, some parasites may employ mechanisms to inhibit eosinophil recruitment, to escape cell-mediated cytotoxicity and to prolong their survival in the host. For example, metalloproteases in the excretory/secretory products of the hookworm *Necator americanus* rapidly cleave eotaxin to prevent eosinophil assemblage (Culley et al. 2000). In another study of ours, bioactivity of a serine-type protease was detected in the excretory/secretory products of *A. cantonensis* worm (C.-M. Yen et al., unpublished data). In our study, proteolysis of eotaxin by *A. cantonensis* did not occur because high concentrations of eotaxin were detectable in the CSF of the infected mice.

There is still no conclusive evidence on whether or not the intensity of immune response is positively correlated with the intensity of the host infection. Reale et al. (1998) reported that the release of the chemokine monocyte chemotactic protein-1 was infectious-dose dependent in mice infected with *Trichinella spiralis*. In our study, when infected mice were divided into four different infection-intensity groups according to worm recovery in the brain, as shown in Table 2, the higher the infectious load, the higher the eotaxin production. However, eotaxin production did not increase further in mice with >30 worms recovered from the brain, compared to that of mice with <30 worms recovered. One of the possible reasons for this might be because the greater worm load leads to more severe eosinophilic meningitis and meningoencephalitis, which causes anorexia and promotes loss of body weight (Wang et al. 1995). Host immune response suffers when host health and nutritional status decline. When we analysed the correlation between CSF eotaxin concentration and infection intensity of each mouse infected with *A. cantonensis*, it showed an absolutely positive significant correlation with worm burdens of <30.

In our study, we not only showed significantly higher eotaxin concentrations in the CSF of mice infected with *A. cantonensis*, but also observed that the release of eotaxin was time-dependent after infection. The increase in eosinophils moving into the CSF paralleled the kinetics of eotaxin concentration change in the CSF of infected mice. Furthermore, the eotaxin concentration in the CSF of infected mice was worm-load dependent if the worm recovery was under a certain threshold (<30 worm). Thus, eotaxin played an important role in the recruitment of eosinophils toward *A. cantonensis* in the brains and spinal cords of infected mice in this study.

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