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Eotaxin Is Specifically Cleaved by Hookworm Metalloproteases Preventing Its Action In Vitro and In Vivo

Fiona J. Culley, Alan Brown, Dolores M. Conroy, Ian Sabroe, David I. Pritchard, and Timothy J. Williams

Eotaxin is a potent eosinophil chemoattractant that acts selectively through CCR3, which is expressed on eosinophils, basophils, mast cells, and Th2-type T cells. This arm of the immune system is believed to have evolved to control helminthic parasites. We hypothesized that helminths may employ mechanisms to inhibit eosinophil recruitment, to prolong worm survival in the host. We observed that the excretory/secretory products of the hookworm Necator americanus inhibited eosinophil recruitment in vivo in response to eotaxin, but not leukotriene B4, a phenomenon that could be prevented by the addition of protease inhibitors. Using Western blotting, N. americanus homogenate was shown to cause rapid proteolysis of eotaxin, but not IL-8 or eotaxin-2. N. americanus homogenate was fractionated by gel filtration chromatography, and a FACS-based bioassay measured the ability of each fraction to inhibit the activity of a variety of chemokines. This resulted in two peaks of eotaxin-degrading activity, corresponding to ~15 and 50 kDa molecular mass. This activity was specific for eotaxin, as responses to other agonists tested were unaffected. Proteolysis of eotaxin was prevented by EDTA and phenanthroline, indicating that metalloprotease activity was involved. Production of enzymes inactivating eotaxin may be a strategy employed by helminths to prevent recruitment and activation of eosinophils at the site of infection. As such this represents a novel mechanism of regulation of chemokine function in vivo. The existence of CCR3 ligands other than eotaxin (e.g., eotaxin-2) may reflect the evolution of host counter measures to parasite defense systems. The Journal of Immunology, 2000, 165: 6447–6453.

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Hookworms currently infect approximately one-fifth of the human population. They reside in the gut with their mouthparts attached to the mucosal surface, feeding on the released blood, where individual worms may persist for years (21). Hookworms are one of the leading causes of anemia in humans, resulting in retardation of physical and mental development in children when infection intensities are severe. A number of nematode secretions have been described that exhibit potent antiinflammatory activities. For example, neutrophil-inhibitory factor (NIF) is a novel class of glycoprotein secreted by Ancylostoma caninum, which exhibits selective and high affinity binding to the integrin CD11b/CD18, an important effector molecule in leukocyte recruitment and activation (22). NIF can block the adhesion of neutrophils to vascular endothelium and their generation of H₂O₂; and in vivo NIF can prevent neutrophil-dependent lung injury (23). Platelet-activating factor (PAF) is a potent stimulator of eosinophil and neutrophil recruitment and activation, and enhances IgE-mediated killing of helminths by eosinophils (24). The parasite Nippostrongylus brasiliensis secretes a PAF acetylhydrolase that cleaves PAF to an inactive form (25).

It is interesting to note that despite apparent activation of peripheral immune responses, localized inflammation is minimal at the site of infection with parasites adapted for the human host, such as Necator americanus, whereas infection with parasites adapted to other hosts, such as Ancylostoma caninum or Antisakia simplex, is associated with pathogenic eosinophilic enteritis (26–28). We reasoned that helminth parasites may have evolved strategies of immune evasion that target the mediators that recruit eosinophils to the site of infection, thus enabling worm survival in the face of host immunity. In this study, we tested the excretory/secretory products of the hookworm N. americanus for the ability to down-regulate recruitment of eosinophils in response to chemotactic agents.

Materials and Methods

Production of N. americanus excretory/secretory products (NES)

The excretory/secretory products of adult N. americanus were collected as previously described (29). Briefly, N. americanus was maintained in DSN hamsters (Mesocricetus auratus), by percutaneous injection of neonates with 100 L3 larvae. Adult worms were recovered from the intestines on day 35. Following extensive washing, 30–35 adult worms were incubated for 16 h in 5 ml RPMI containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Paisley, U.K.). To standardize activity, each batch of NES produced was assessed for fibrinogenolytic protease activity, as previously described, and concentrated so that 20 μl NES digested 10 μg fibrinogen in 4 h (30). Bacterial contamination of NES was absent, as determined by culture of aliquots on 1-agar plates and incubation overnight at 37°C.

Protease inhibitors

The following inhibitors of protease activity were used (Sigma, Poole, U.K.): pepstatin A (aspartic proteases), phenanthroline (metalloproteases), trans-epoxy succinyl- l- leucylamido (4-guanidino) butane (E-64) (cysteine proteases), PMSF (serine proteases), EDTA (metalloproteases and calcium-dependent proteases), and EGTA (calcium-dependent proteases). Pepstatin A, PMSF, phenanthroline, and E-64 are known to inhibit the four classes of proteases released by N. americanus (30).

In vivo bioassay of 111In-labeled eosinophil accumulation

The recruitment of eosinophils in response to inflammatory mediators in vivo was measured using a skin bioassay, as previously described (31). Briefly, eosinophils were purified from the peritoneal exudate of donor guinea pigs, which had been induced by repeated injection of horse serum. Eosinophils were radiolabeled with 111IICl₃ (Amersham Life Science, Little Chalfont, U.K.), and injected i.v. into sedated recipient animals. For each injection site, 60 nM guinea pig eosinatin (generic gift from H. Showell, Pfizer, Groton, CT) or 600 nM leukotriene B₄ (LTB₄) in 50 μl HBSS incubated with an equal volume of NES or buffer control, in the presence or absence of protease inhibitors (1 μM pepstatin A, 1 mM phenanthroline, 1 μM E-64, and 0.1 mM PMSF), incubated for 1 h at room temperature, then injected intradermally into the dorsal skin of recipient animals. Injections were performed at duplicate sites on each of four recipient animals per experiment, and the experiment was conducted three times. After 2 h, animals were killed, the dorsal skin removed, the skin injection sites punched out, and 111In-labeled eosinophil accumulation measured using a Cobra gamma counter (Packard Bioscience, Pannsbourne, U.K.). Cell recruitment was averaged for the two injection sites on each animal and data expressed as the mean ± SEM of the four animals in each group. The statistical significance of the data was analyzed using the Student’s t test.

Eotaxin ELISA

A total of 25 μl NES was mixed with 50 pM guinea pig or human eotaxin (PeproTech, London, U.K.) in the presence of absence of the protease inhibitor cocktail detailed above, in a final volume of 100 μl PBS. After incubation for 1 h at 37°C, 100 μl of protease inhibitor cocktail in PBS was added to all reactions. Immuno reactive eotaxin was measured by specific sandwich ELISAs developed in our laboratories, as previously described (32, 33).

Western blotting of human chemokines

Western blotting was used to assess the time course and specificity of chemokine digestion by NES proteases. A total of 20 pmol of human eotaxin, eotaxin-2, or IL-8 (PeproTech), in 2 μl 0.1% BSA, PBS was mixed with 10 μl of NES and incubated at 37°C. For each chemokine, 10, 5, and 2.5 pmol of chemokine were also incubated in 12 μl PBS to provide a standard curve. The reaction was stopped by the addition of an equal volume of 2× SDS-PAGE sample buffer immediately boiling for 5 min. Samples were resolved on a 15% SDS-PAGE gel under standard conditions (34). Proteins were transferred onto sequencing grade polyvinylidene difluoride membranes (Millipore, Watford, U.K.) in 25% methanol, 25 mM Tris base, 200 mM glycine, and 1.7 mM SDS for 1 h. Membranes were blocked with 5% fat-free milk powder in PBS for 30 min, followed by incubation overnight at 4°C with primary anti-chemokine Ab at 1:10,000 in 0.1% milk powder in PBS under constant agitation. We used the IgG fraction of rabbit anti-human eotaxin Ab N3, prepared from rabbit serum by precipitation of unwanted proteins with caprylic acid, as described (35, 36). Eotaxin-2 was detected using the mouse anti-human eotaxin-2 Ab 9G6 (generic gift from Dr. Paul Ponath, Leukosite, Cambridge, MA), and IL-8 detected using mouse anti-human IL-8 Ab (R&D Systems, Abingdon, U.K.). Membranes were washed for 3 × 5 min in 0.05% Tween 20, PBS, followed by detection with peroxidase-conjugated secondary Ab at 1:10,000 in 0.1% milk powder in PBS for 1 h at room temperature. Membranes were washed as above and developed using chemiluminescent substrate (Pierce and Warriner, Chester, U.K.) and x-ray film (X-ohraph).

Preparation of N. americanus homogenate

Adult worms were homogenized on ice in PBS using a polytron (Kine- matica, Lucerne, Switzerland). Insoluble material was removed by centrifugation at 13,000 × g for 10 min and the supernatant retained. Before gel filtration, glycerol was added to the sample to a final concentration of 5%.

S-300HR gel filtration of N. americanus homogenate

Gel filtration was conducted using the low pressure Bio-Rad (Hemel Hempstead, U.K.) column system. A glass chromatography column (50 cm × 50 cm) was packed with Sephacryl S-300 HR and equilibrated with PBS (flow rate 0.33 ml/min). The column was calibrated and the void volume determined using broad range gel filtration standards (200–12.5 kDa; Sigma, Poole, U.K.). A total of 2 ml of adult homogenate (~5 mg protein) was applied to the column, and following the elution of the void volume, 2-ml fractions were collected and assayed for their ability to degrade eotaxin.

Measurement of chemokine bioactivity following incubation with homogenate fractions

A total of 25 μl of each fraction was mixed with 75 μl 3 nM eotaxin, eotaxin-2, IL-5 or IL-8, 0.1% BSA, 10 mM HEPES, PBS, and incubated for 1 h at 37°C to allow digestion to take place. Remaining bioactivity on eosinophils and neutrophils was measured using a FACS-based assay of...
cell shape change (37). Granulocytes were prepared from the blood of volunteers who were healthy or had asthma, eczema, or hay fever, according to the following technique (under the approval of St Mary’s Hospital Local Ethics Committee). Cells were separated from plasma by centrifugation of citrated whole blood, and the erythrocytes were removed by dextran sedimentation. Monocytes and granulocytic leukocytes were separated over a Histopaque density gradient, and any remaining erythrocytes were removed by hypotonic shock lysis. For the assay, $5 \times 10^5$ mixed granulocytes were added to the agonist and incubated at 37°C for 6 min, after which the cells were fixed. A FACScan flow cytometer (Becton Dickinson, Mountain View, CA) was used to separate eosinophils and neutrophils based on their autofluorescence measured in the FL-2 channel. Cell stimulation produces a shape change that is measured as an increase in mean forward scatter, and that is in proportion to the concentration of agonist. Experiments were performed on cells from a minimum of three donors. Using the ANOVA test, the response produced in the presence of each fraction was compared with the response produced by chemokine alone.

**Use of selective protease inhibitors to determine the class of protease acting on eotaxin**

Eotaxin was incubated with *N. americanus* gel filtration fractions in the presence of a range of protease inhibitors, to determine the class of protease(s) responsible for chemokine digestion. Following incubation, immunoreactive eotaxin was measured by ELISA. A total of 12.5 μl of each fraction was incubated with 50 pM eotaxin in the presence or absence of protease inhibitors in a final volume of 100 μl 0.1% BSA, PBS. The following inhibitors were used: 20 mM EDTA, 1 mM EGTA, 1 μM pepstatin A, 10 μM E-64, 10 mM phenanthroline, 1 mM PMSF. Controls containing protease inhibitors and eotaxin, but no fraction, were included to ensure that the protease inhibitors did not affect the measurement of eotaxin by the ELISA. Samples were incubated for 1 h at 37°C, followed by cooling on ice and the addition of 150 μl of ice-cold 0.1% BSA, PBS. Eotaxin was measured by ELISA, as described above.

**Results**

Necator-derived proteases prevent eotaxin-mediated eosinophil recruitment in vivo

To determine whether the excretory/secretory products of *N. americanus* could inhibit the recruitment of eosinophils in vivo, we preincubated two mediators of eosinophil recruitment, eotaxin and LTB$_4$, with NES and measured their activity in an in vivo bioassay. The chemoattractants were injected intradermally into recipient guinea pigs, which had previously received radiolabeled eosinophils i.v. Eosinophil recruitment was determined by counting the radioactivity at each injection site after 2 h. As shown in Fig. 1, both eotaxin and LTB$_4$ stimulate the recruitment of eosinophils to the site of injection. Preincubation of eotaxin with NES significantly reduced the recruitment of eosinophils in response to this agonist, but had no effect on LTB$_4$-mediated recruitment. The effect of NES on eotaxin could be prevented by the addition of a cocktail of protease inhibitors (phenanthroline, E-64, pepstatin A, and PMSF) that has been previously shown to inhibit all classes of protease activity in NES. The protease inhibitors had no effect on eosinophil recruitment when injected alone or in combination with LTB$_4$, and NES did not induce eosinophil recruitment when injected alone (Fig. 1). Therefore, we conclude that *N. americanus* produces proteases that are able to inhibit eosinophil recruitment in vivo.

**Necator proteases cause loss of eotaxin immunoreactivity**

We investigated the effect of NES on eotaxin immunoreactivity. Both guinea pig and human eotaxin were incubated with NES in the presence, or absence, of protease inhibitors and the remaining immunoreactive chemokine measured by ELISA. As shown in Fig. 2, incubation of both guinea pig and human eotaxin with NES reduced the eotaxin measurable by ELISA. The addition of protease inhibitors reversed this, demonstrating that proteolysis was responsible for the loss of immunoreactivity. The protease inhibitors themselves had no effect on the ELISA system (data not shown).

**Time course and specificity of proteolysis of eotaxin**

To determine whether the excretory/secretory products of *N. americanus* could inhibit the recruitment of eosinophils in vivo, we preincubated two mediators of eosinophil recruitment, eotaxin and LTB$_4$, with NES and measured their activity in an in vivo bioassay. The chemoattractants were injected intradermally into recipient guinea pigs, which had previously received radiolabeled eosinophils i.v. Eosinophil recruitment was determined by counting the radioactivity at each injection site after 2 h. As shown in Fig. 1, both eotaxin and LTB$_4$ stimulated the recruitment of eosinophils to the site of injection. Preincubation of eotaxin with NES significantly reduced the recruitment of eosinophils in response to this agonist, but had no effect on LTB$_4$-mediated recruitment. The effect of NES on eotaxin could be prevented by the addition of a cocktail of protease inhibitors (phenanthroline, E-64, pepstatin A, and PMSF) that has been previously shown to inhibit all classes of protease activity in NES. The protease inhibitors had no effect on eosinophil recruitment when injected alone or in combination with LTB$_4$, and NES did not induce eosinophil recruitment when injected alone (Fig. 1). Therefore, we conclude that *N. americanus* produces proteases that are able to inhibit eosinophil recruitment in vivo.

**Time course and specificity of proteolysis of eotaxin**

Human eotaxin, eotaxin-2, and IL-8 were incubated with NES for 30, 60, or 90 min, and proteolysis was determined using Western blotting. Following incubation, chemokines were resolved on a 15% acrylamide SDS-PAGE gel, transferred to a polyvinylidene difluoride membrane, and detected using specific Abs. For each chemokine, a standard curve was run on the same gel to show the reduction in signal in proportion to reducing amounts of chemokine. Eotaxin was rapidly degraded in the presence of NES (Fig. 3). No eotaxin degradation products could be detected by the Ab. Under the same conditions, IL-8 showed no loss of signal even after 90 min of incubation at 37°C. In a series of three experiments,
eotaxin-2 showed no, or very slight, loss of immunoreactivity when incubated with NES for 90 min. Therefore, *N. americanus* produces protease activity that exhibits substrate specificity for eotaxin.

*N. americanus* protease activity specifically inhibits eotaxin bioactivity

To characterize the functional consequences and specificity of hookworm-derived protease activity further, adult *N. americanus* homogenate underwent gel filtration chromatography. Each fraction was tested for its ability to inhibit chemokine activity in an in vitro bioassay. Chemokines were preincubated with homogenate fractions for 1 h at 37°C and then added to mixed granulocytes that had been freshly isolated from human blood. Bioactive chemokines produce a shape change in responsive cells that can be measured as an increase in forward scatter on a flow cytometer (37).

We measured the activity of eotaxin, eotaxin-2, and IL-5 on eosinophils, and of IL-8 on neutrophils. Fig. 4 shows the activity of each chemokine measured as a change in forward scatter. Inhibition of eotaxin bioactivity occurred in two major peaks at fractions 9–12 and 14–17 (*p* < 0.01, ANOVA comparison with maximal response), peaking at fractions 11 and 16, which correspond to molecular mass of ~15 and 50 kDa. In contrast, the same fractions did not significantly alter the shape change response to IL-8, IL-5, and eotaxin-2, compared with the maximal response (*p* > 0.05 in all fractions).

Eotaxin digestion is prevented by metalloprotease inhibitors

*N. americanus* produces all of the four major classes of proteases: metallo-, cysteine, aspartic, and serine proteases. To determine which classes of enzymes were involved in the proteolysis of eotaxin, we incubated eotaxin with homogenate fractions, in the presence of individual protease inhibitors. In preliminary experiments, protease inhibitors were found to interfere with the shape change assay of eosinophil activation. Therefore, immunoreactive eotaxin was measured using an ELISA. As shown in Fig. 5, the fractions that show the most anti-eotaxin activity by ELISA, i.e., fractions 11 and 16, correspond to those that are the most inhibitory in the bioassay shown in Fig. 4. All eotaxin-degrading activity was prevented by the addition of EDTA, suggesting that the activity was dependent upon divalent cations. Both peaks of activity were present in EGTA-treated samples, which selectively chelate calcium, suggesting that the activity is calcium insensitive. The specific metalloprotease inhibitor, phenanthroline, inhibited activity in both peaks, suggesting that metalloprotease activity was responsible for the major activity seen. In addition, incubation of fractions with the metalloprotease substrate Suc-Gly-Pro-Leu-Gly-Pro-AMC, indicated that metalloproteases eluted between fractions 9 and 18, the same region in which eotaxin degradation occurs (A. B., unpublished data). The aspartyl protease inhibitor pepstatin A inhibited some activity between these two peaks (fractions 12–15), suggesting that there may be other protease activity present that can digest eotaxin, albeit with lower activity than the major metalloprotease activities seen.
FIGURE 5. Characterization of eotaxin-degrading activity of hookworm homogenate. The graphs show a typical experiment, of three, in which eotaxin was incubated with fractions of homogenate in the presence of protease inhibitors and remaining eotaxin was measured by ELISA. Two major peaks of activity are apparent in the fractions incubated in the absence of protease inhibitors. The experiment was repeated in the presence of the following inhibitors: E-64, EDTA, EGTA, phenanthroline, PMSF, pepstatin A. Both of the major peaks of activity are inhibited by EDTA and phenanthroline, suggesting that the eotaxin degradation is due to the actions of a metalloprotease.

Discussion

Little is known about mechanisms involved in chemokine regulation in vivo and potential manipulations by foreign organisms to evade host defense. The mammalian protease CD26 cleaves all chemokines containing the NH₂-X-Pro motif. Both mammals and viruses encode apparently nonsignaling receptors that may sequester chemokines, preventing their actions on target cells. In addition, proteoglycans that can bind all chemokines, both at the cell surface and as part of the extracellular matrix, may be important regulators of chemokine function (38, 39).

We have demonstrated that the adult hookworm *N. americanus* produces proteases that can cleave eotaxin. Following the action of these proteases, eotaxin can no longer be detected in immunosays and exhibits no activity on eosinophils in both in vitro assays of eosinophil shape change and in an in vivo assay of eosinophil recruitment. These proteases demonstrate a specificity for eotaxin, as they do not prevent the actions of LTB₄, IL-5, or eotaxin-2 on eosinophils or IL-8 on neutrophils. Following gel filtration chromatography of adult worm homogenate, two major peaks of eotaxin-degrading activity were detected. These peaks were inhibited by the addition of EDTA and the specific metalloprotease inhibitor phenanthroline, but not EGTA or any of the other classes of protease inhibitors tested, suggesting that eotaxin is cleaved by calcium-independent metalloproteases. The two peaks of activity may represent two distinct proteases or different molecular mass forms of the same protease, such as glycosylation variants or multimers. The products of *Necator* are a crude mixture that is known to contain a number of different proteases, with different pH dependencies (30). It will be interesting to discover whether other products of *N. americanus* exhibit anti-inflammatory properties, under different conditions. Purification of the metalloprotease we describe in this work will allow a fuller characterization of the enzyme’s substrate specificity and biochemistry.

It has long been a puzzle as to how parasites establish chronic infection in the host despite the induction of a strong immune response. To survive, helminth parasites secrete a number of factors that are capable of down-regulating host immune attack. Hookworms secrete NIF, which may block recruitment of inflammatory cells; glutathione-S-transferase, an antioxidant that may neutralize toxic oxygen products of eosinophils; an Igase, which may prevent Ab-mediated immune attack; an acetylcholinesterase, which may inhibit gut contraction; and a hyaluronidase that may facilitate the spreading of these mediators into the surrounding tissue by digesting the extracellular matrix (22, 40–43). In addition, *N. americanus* secretions have recently been shown to induce apoptosis in T lymphocytes (44). The activity we describe in this study is likely, therefore, to function in the context of a protective defense shield secreted by the parasite. Helminths have evolved manifold mechanisms of inhibiting various stages of the host attack: cellular recruitment, cell activation, and the actions of toxic cellular products and mediators that induce expulsion of the parasites from the gut (reviewed in Ref. 45). A well-adapted parasite causes morbidity, allowing for chronic production of eggs, rather than mortality, which is likely to occur in a host overburdened with parasite infection or severely immunocompromised. Rather, the parasite and host exist in equilibrium. In reply to the immunosuppressive adaptations of the parasites, the host may evolve new mechanisms of attack. In this respect, it is interesting that the eotaxin-digesting protease activity we describe cannot inactivate eotaxin-2, under the conditions used. Eotaxin-2 has only 39% identical amino acids to eotaxin; however, its activity on eosinophils is indistinguishable from that of eotaxin, signaling selectively via CCR3. It is notable that several other C-C chemokines signal via the same receptor CCR3, for reasons that are unclear. Evolutionary pressure on CCR3 ligands exerted by host defense evasion strategies developed by parasites provides a possible explanation. Proteolysis of eotaxin, as described in this study, may be an example of this phenomenon.

Parasitic helminths secrete a wide range of proteases that play important roles in their life cycles, such as invasion of host tissue and feeding. *N. americanus* secretes aspartyl, cysteiny1, serine, and...
metalloproteases. These proteases have been found to exhibit fibrinolytic and hemoglobinase activity, which would prevent blood clotting at the bite site and facilitate digestion of blood (30, 46). As parasite proteases are often essential for, and unique to the parasitic lifestyle, they represent attractive targets for chemotherapy and vaccine development. For example, vaccination of sheep with a multiprotease-containing complex from the intestine of the nematode *Haemonchus contortus* results in greatly reduced worm burdens and egg output associated with anti-protease Abs (47).

The discovery of proteases that are important virulence factors for parasitic disease may result in the rational design of specific inhibitors as chemotherapeutic agents (48). For example, *Schistosoma mansoni* secretes a cysteine protease that allows these parasites to digest hemoglobin, a major protein source for their metabolism. Inhibition of cysteine proteases in vivo in murine models of infection resulted in a significant reduction in worm burden, hepatomegaly, and fecundity (49). One precedent for clinical use of protease inhibitors as chemotherapy against infection is in HIV infection, in which they have proved effective at reducing viral levels and delaying the onset of AIDS. In this case, inhibitors are directed against aspartic proteases involved in viral particle assembly. The development of effective agents for the specific inhibition of nematode infection is imperative, as these parasites represent major burdens not only on human health, but also financially on agriculture. The antitoxin proteases we describe in this study are particularly attractive targets for chemotherapy because of their apparent substrate specificity, and may prove to be effective Ags for vaccination against infection.

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