Zinc and Manduca sexta hemocyte functions

Authors: Willott, Elizabeth, and Tran, Hung Q.

Source: Journal of Insect Science, 2(6) : 1-9

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.002.0601

Willott E and Tran HQ. 2002. Zinc and *Manduca sexta* hemocyte functions. 9 pp. *Journal of Insect Science*, 2:6. Available online: insectscience.org/2.6

Journal of Insect Science

insectscience.org

Zinc and *Manduca sexta* hemocyte functions

Elizabeth Willott and Hung Q Tran

Department of Entomology, The University of Arizona, Tucson AZ 85721-0036.
willott@u.arizona.edu

Received 8 January 2002, Accepted 6 March 2002, Published 25 March 2002

Abstract

Two metalloproteases have recently been linked to the immune response in Lepidoptera. In addition, zinc is highly important in many mammalian immune-related functions. Because of these, we investigated the effect of zinc and two zinc-protease inhibitors on *Manduca sexta* hemocyte behavior *in vitro*. Plasmatocytes were significantly more elongated in Grace's medium supplemented with 100 µm zinc chloride than in the absence of zinc. To test whether zinc-dependent proteases were responsible for the increased length seen in the presence of zinc, we tested two zinc-protease inhibitors, phosphoramidon and bestatin. Each resulted in decreased plasmatocyte length compared to the control, but the distributions of lengths differed with each inhibitor. Each inhibitor also affected plasmatocyte network formation *in vitro.* This work suggests (1) that at least two different zinc proteases are involved in the cellular defense response of *M. sexta*, and (2) that zinc should be included in media used for *in vitro* studies of the immune response.

Keywords: cell spreading, tobacco hornworm, divalent cations, plasmatocytes

Abbreviation:

TBS	Tris-buffered saline
T-TBS	Tris-buffered saline containing 0.05% Tween.
AC-saline	*Manduca sexta* anti-coagulant saline
SOD	superoxide dismutase
NEP	neutral endopeptidase

Introduction

An insect defends itself against pathogens and parasites by several processes including phagocytosis, nodule formation, and encapsulation (for reviews, see Ratcliffe, 1993; Strand and Pech, 1995; Gillespie *et al.*, 1997; Trenczek, 1998). Phagocytosis, nodule formation, and encapsulation are multi-stage processes involving many different signaling and effector molecules that are now being identified and characterized.

Divalent cations enhance or are required for phagocytosis, nodule formation, encapsulation, and other immune-related processes of insects (Drif and Brehélin, 1994; Mandato *et al.*, 1996; Bettencourt *et al.*, 1997; Bettencourt *et al.*, 1999; Tojo *et al.*, 2000). The roles of calcium and magnesium have been the most extensively studied. We recently found that calcium is needed for effective elongation of plasmatocytes growing on plastic; calcium also enhances the formation of a hemocyte network *in vitro* (Willott *et al.*, 2002). Study of other divalent cations have been largely neglected presumably in part because media lacking detectable amounts of other divalent cations have not been shown to be deleterious to insect cells. For instance, *Manduca sexta* hemocytes survive for up to 72 hours in Grace's insect medium with undetectable levels of zinc.

Recently zinc-dependent metalloproteases have been linked to the lepidopteran immune response. A zinc-metalloprotease inhibitor was induced in hemolymph of the greater wax moth *Galleria mellonella* following suitable challenge by injection of either bacteria or small (<3 kDa) peptides that were generated by digesting *G. mellonella* hemolymph proteins with thermolysin (Wedde *et al.*, 1998). The inhibitor, an 8.4 kDa protein, has no similarity to known proteins; inhibits bacterial thermolysin, but is also cleavable by thermolysin; has ten cysteines; and appears at approximately the same time as other inducible immune proteins. A fungal pathogen, that infects *G. mellonella*, *Metarhizium anisopliae*, produces a thermolysin-like metalloprotease that penetrates the integument and digests larval proteins. It also inhibits attachment, spreading, and phagocytic activity of plasmatocytes (Griesch and Vilcinskas, 1998). If the host produced the inhibitor of this metalloprotease in response to fungal infection, it could help the larvae resist pathogens

A cDNA clone encoding a putative zinc-dependent protease was recently isolated from *M. sexta* (M. Kanost, personal communication). This clone, obtained by a cDNA subtraction

technique using fat body mRNA from bacteria-injected and control larvae, has sequence similarity to a *Bombyx mori* sequence (AB0428208) and to two *Drosophila* sequences (AAD347431.1 and AAF46125.1). The functions of the proteins encoded by those sequences are not known; however, they all are homologous to mammalian zinc-dependent protease sequences, the closest being the mammalian neutral endopeptidase 24.11 (i.e., neprolysin, or NEP). Sequences of two putative metal-requiring enzymes have been identified by a PCR-based differential display and subtractive cloning technique using RNA from the fall webworm *Hyphantria cunea*, a putative metalloprotease, and a putative copper- and zinc-dependent superoxide dismutase (Cu-Zn SOD) (Shin *et al.*, 1998). Given the evidence for metalloprotease involvement in the immune response of Lepidoptera, the importance of zinc-dependent proteases in the mammalian immune system, and the suggestive evidence that zinc-dependent proteases are important in the insect immune response, we tested zinc and the zinc-protease inhibitors phosphoramidon and bestatin for their effect on *M. sexta* hemocyte behavior *in vitro*.

Materials and Methods

Rearing M. sexta larvae

Day 2 and day 3, fifth instar larvae were used. *M. sexta* eggs were provided by Dr. Michael Wells, The University of Arizona. Larvae were reared as previously reported (Fernando-Warnakulasuriya *et al.*, 1988). They were reared at 21°C, individually in wells of tissue culture plates and then in plastic dessert dishes. Diet was changed every second day for the early instars and daily for fifth instars.

Buffers

AC saline was made as previously described (Willott *et al.*, 1994). Grace's medium came from Gibco BRL. Tris-buffered saline (TBS): 137 mM NaCl, 3 mM KCl, 25 mM Tris, pH 7.0. Microscopy fixative was 5% formaldehyde (made fresh from paraformaldehyde) in TBS, then pH adjusted to pH 6.8.

Determination of zinc concentration in hemolymph: atomic absorption spectroscopy

Three larvae, surface sterilized with ethanol, were bled by inserting a syringe needle tip into the hemocoel. Freely dripping hemolymph was collected into chilled microfuge tubes and 50 µl was immediately diluted into buffer and analyzed on an IL atomic absorption spectrometer. Results were 8.8, 8.0, and 4.6 ppm giving an average of 7.13 ppm or approximately 100 µM. Grace's insect medium (Gibco) showed no detectable zinc (<0.05 ppm).

Preparation of hemocytes

For each experiment, six larvae (3 male and 3 female), surface sterilized with ethanol, were bled by inserting a syringe needle tip into the hemocoel. Freely dripping hemolymph was collected into ice cold anticoagulant saline (AC saline) (Willott *et al.*, 1994). To represent all animals appropriately, the equivalent of 100-150 µl of hemolymph from each larva was used. Hemolymph was centrifuged at 100*g* for 10 min at 4°C. Supernatant was discarded and hemocyte pellets were used for the experiments outlined below.

Plasmatocyte spreading and network formation

Initial study

Hemocyte pellets, obtained as described above, were resuspended and pooled in a total of 1.5 ml Grace's medium. Hemocyte solution (for final $2.3x10^5$ cells/cm^2) was added to each well of a 12-well Nunclon∆ multidish (Nunc, Inc., Naperville, IL). Wells contained (a) Grace's medium (no zinc); (b) Grace's medium with 100 µM zinc; (c) Grace's medium with 100 µM zinc and 20 mM EGTA; (d) Grace's medium with 100 µM zinc and 100 µM phosphoramidon (Sigma R7385; recommended concentration is 7-570 µM see Roche Molecular Biochemicals http://biochem.roche.com); (e) Grace's medium with 100 µM zinc and 1 µM bestatin (Sigma B3585; recommended concentration [see Roche] is 1 µM). Hemocytes were incubated at 29 °C, and were photographed at 2, 5, 8.5, 20, and 26 h. At 26 h, cells were fixed by adding 150 µl of fixative and incubating 10 min. Fixative was removed and TBS containing 0.05% thimerosol was added to the wells.

Plasmatocyte length and width

Plasmatocyte length was defined as the length of the long axis through the cell; width was the short axis taken through the nucleus. An example of the measuring is shown in red in Fig. 1. Measures were made with Adobe Photoshop (http://www.adobe.com/products/photoshop/main.html) of images taken with a digital camera, with the slide containing the cells still in place on the microscope so the edges of cells could be confirmed by adjusting the fine focus when necessary. In our previous work on calcium, we found that some plasmatocytes never spread. In that work, plasmatocytes were identified by immunofluorescence microscopy using the plasmatocyte specific monoclonal antibody MS13 (Willott *et al.*, 1994). Even in the presence of zinc, some plasmatocytes remained unspread. Because we were using the Nunclon∆ plates rather than glass slides, we initially identified plasmatocytes by immunocytochemistry using plasmatocyte-specific monoclonal antibody MS13 (Willott *et al.*, 1994). After fixation, cells were rinsed once with TBS, then non-specific binding sites were blocked with 2% bovine serum albumin (BSA) in TBS containing 0.05% Tween (T-TBS). BSA-T-TBS was removed and to each well was added 0.8 ml of hybridoma supernatant containing monoclonal antibody MS13. Plates were incubated 1 h, wells rinsed 3 times with TBS, incubated in secondary antibody (1:500 dilution of goat anti-mouse antibody-hydrogen peroxidase conjugate; Life Technologies 13871-017) for 1 h, rinsed 3 times with TBS and incubated 20 to 30 min in 3-amino-9-ethylcarbazole (AEC) commercial color development reagent (Vector Laboratories, CA; SK-4200). Wells were rinsed once with TBS, then TBS with the preservative thimerosol was added. Unspread plasmatocytes were approximately 7-10 µm in length. To reduce any error associated with losing cells during the washes of the staining procedure, we decided to forego immunostaining, and score only plasmatocytes with length greater than 15 µm since such plasmatocytes were easily identifiable by phase-contrast microscopy. Other researchers (Griesch and Vilcinskas, 1998; Strand and Clark, 1999) have assayed plasmatocytes defined in terms of length,.

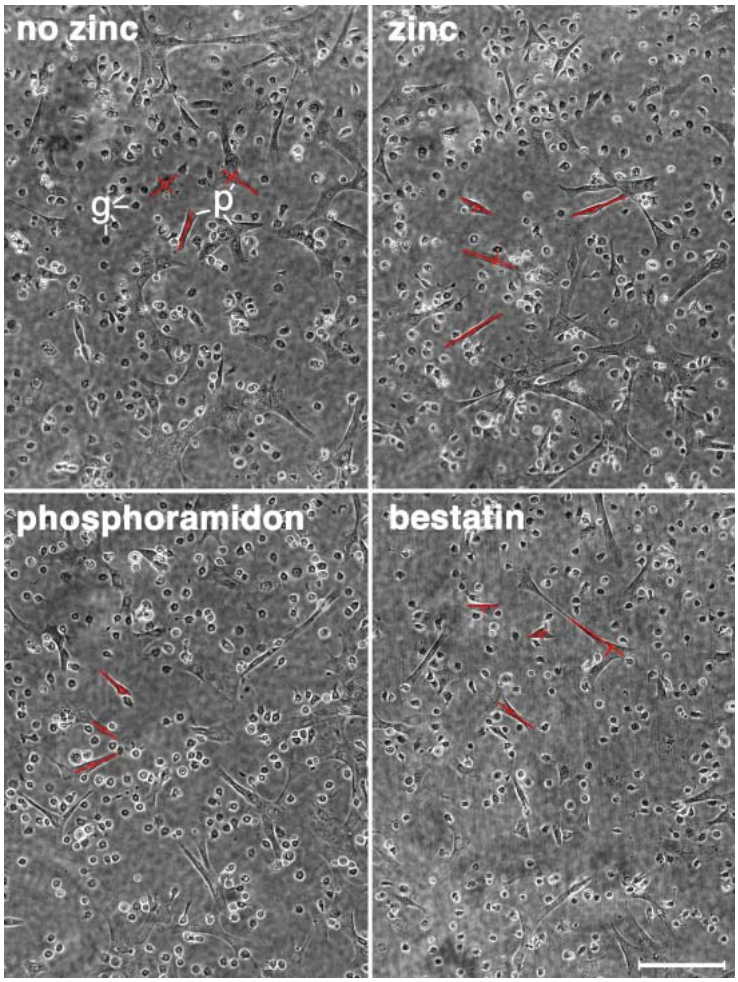

Figure 1. Phase contrast micrographs of hemocytes incubated 18 h on NunclonΔ 96-well tissue culture plates. 5 x 10^5 hemocytes/well. P = plasmatocytes; g = granular cell. (a) 0 µM zinc; (b) 100 µM zinc; (c) 2.5 µM phosphoramidon and 100 µM zinc, (d) 1 µM bestatin and 100 µM zinc. These cells are some of those measured for #4 data in Tables 1 and 2. A sample of the cells are labeled in red to indicate how cell measurements were done. Bar = 100 µm.

Plasmatocyte spreading and network formation on 96-well NunclonΔ plastic dishes

Hemocyte solution, prepared as above, was added to each well of NunclonΔ 96-well tissue culture plates (Nalge Nunc International Corporation, Naperville, IL) containing 100 µl of Grace's medium plus relevant ions, EGTA, or protease inhibitors as indicated above. For measuring cell length, hemocytes were plated at a density of 5-7 x 10^4 cells/well. For observing network formation, hemocytes were plated at 1-1.5 x 10^5 cells/well. Plates were incubated at 27 °C for 17 h. Cells were fixed by adding 100 µl of fixative and incubating 10 min. Fixative was removed and TBS with thimerosol was added to the wells. To quantify differences arising from the treatments, the lengths and widths of 50 plasmatocytes were measured per treatment for each of four to six replicates (i.e., a total of 200-300 cells were measured per treatment).

Photography

Photographs were taken with a Nikon Eclipse E600 microscope with a SPOT RT color camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and analyzed with SPOT RT software (Diagnostic Instruments, Inc.) and Adobe PhotoShop (Adobe Systems, Inc., San Jose, CA).

Statistical treatment of the data

Length and width data were analyzed by least squares analyses of variance using the General Linear Model of SAS (1987). Data from replicate #4 of Tables 1 & 2 is illustrated using a scatterplot to show variation in plasmatocyte shape for a given treatment and to show the proportion of plasmatocytes of a given shape for each treatment.

Results

Zinc and the zinc-protease inhibitors phosphoramidon and bestatin had a significant effect on *M. sexta* hemocytes incubated *in vitro*. When plated at low cell density (i.e., ~5 x 10^4/well of a 96-well plate or ~1.5 x 10^5/cm^2) *M. sexta* hemocytes were noticeably affected by the presence or absence of zinc or either of the zinc-protease inhibitors. In the presence of zinc (and absence of inhibitors), plasmatocytes were more elongated and more interconnected (Figure 1).

Table 1. Effect of zinc, phosphoramidon, and bestatin on plasmatocyte length

	Length (µm)[a]			
	0 µM Zn	100 µM Zn	Phosphoramidon [b]	Bestatin[b]
#1	36.1 ± 13.1 (18.4-82.7)	44.0 ± 16.9 (21.3-100.7)	******	******
#2	37.2 ± 11.3 (18.1-74.0)	41.4 ± 13.6 (20.6-92.8)	******	******
#3	41.5 ± 12.9 (19.1-82.7)	47.4 ± 14.8 (18.4-78.0)	37.3 ± 11.8 (20.9-80.7)	40.4 ± 11.6 (19.5-84.1)
#4	41.1 ± 9.7 (25.3-66.1)	43.1 ± 11.5 (23.5-81.6)	38.8 ± 8.23 (37.9-50.2)	35.7 ± 13.6 (19.9-78.0)
#5	******	41.4 ± 10.4 (24.9-69.0)	36.7 ± 10.7 (22.7-78.7)	38.0 ± 10.7 (19.5-72.9)
#6	******	43.87 ± 17.6 (18.4-93.1)	29.67± 7.5 (17.3-43.7)	33.61± 9.6 (15.9-57.0)

a) Mean ± SD. N e" 50 for each test treatment. Range given in brackets.
b) 100 µM zinc was also present.

0 µM zinc vs. 100 µM zinc: #1-4 (uneven dashed box)

0 µM zinc vs. 100 µM zinc	p < 0.0002

100 µM zinc vs. bestatin or phosphoramidon: #3-6 (even dashed box)

Bestatin vs. 100 µM zinc	p < 0.0001
Phosphoramidon vs. 100 µM zinc	p < 0.0001
Phosphoramidon vs. bestatin	p = 0.25

All factors co-analyzed: #3 & #4 (solid line)

0 µM zinc vs. 100 µM zinc	p = .021
Bestatin vs. 100 µM zinc	p < 0.0001
Bestatin vs. 0 µM zinc	p = 0.055
Phosphoramidon vs. 100 µM zinc	p <.0001
Phosphoramidon vs. 0 µM zinc	p = 0.052
Phosphoramidon vs. bestatin	p = 0.98

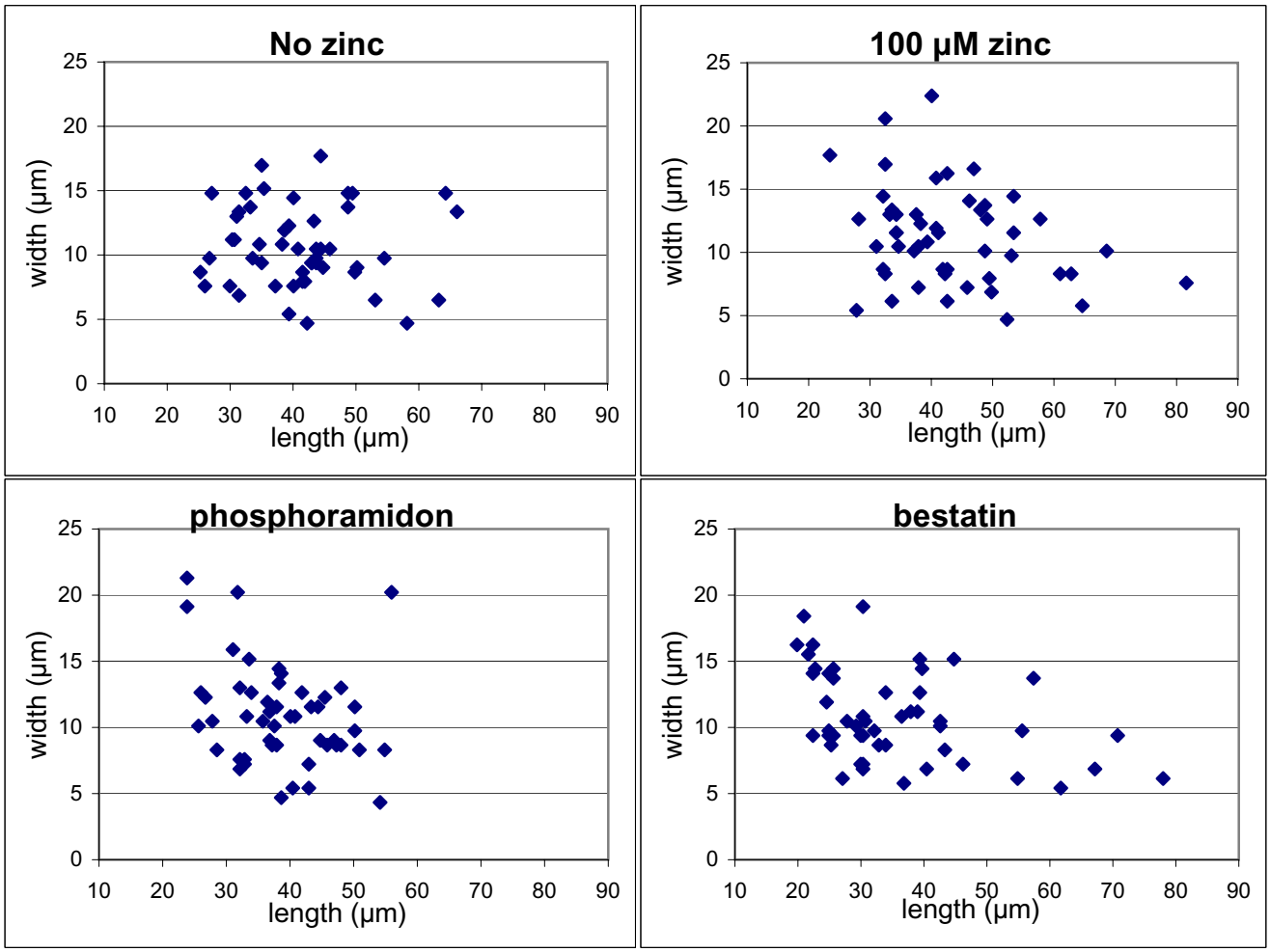

Figure 2. Scatter plots of length and width of plasmatocytes incubated 18 h on NunclonΔ 96-well tissue culture plates. Each point represents the length (x-axis) and width (y-axis) of an individual spread plasmatocyte in Grace's insect medium supplemented with (a) 0 µM zinc; (b) 100 µM zinc; (c) 2.5 µM phosphoramidon and 100 µM zinc; (d) 1 µM bestatin and 100 µM zinc. These cells are some of those measured for #3 data in Tables 1 and 2.

Mean plasmatocyte length was greatest for plasmatocytes in the presence of 100 µM zinc, and absence of inhibitors (Table 1). Addition of either bestatin or phosphoramidon, in the presence of 100 µM zinc, resulted in mean lengths similar to those obtained in the absence of zinc. The differences between these treatments and zinc were statistically significant. Plasmatocyte width was not as strongly affected (Table 2) with only zinc compared to no zinc showing statistical significance.

The scatterplot (Figure 2) of the length and width of individual plasmatocytes indicates plasmatocytes responded differentially to phosphoramidon and bestatin. Plasmatocytes in phosphoramidon-containing solutions did not extend past 55 µm, with most being between 30 and 50 µm. In contrast, in bestatin, a sizable proportion of the plasmatocytes were below 35 µm in length, while the lengths of the remainder were comparable to lengths reached in the presence of zinc. A systematic effect on plasmatocyte width was not apparent.

Formation of networks was cell concentration dependent (Figure 3). When plated at high densities (e.g., e"1.3 x 10^5/well or higher) plasmatocytes readily connected with each other and formed extensive networks. This would happen regardless of the presence or absence of zinc or the presence of either inhibitor plus zinc (data not shown).

When hemocytes were plated at moderate densities (e.g., ~1.0 x 10^5) different treatments notably affected network formation (Figure 4). In the presence of zinc, the network formed had regularly spaced nodes (interconnecting sites) and approximately the same number of plasmatocytes per node. Plasmatocytes also aligned with one another in an organized way. In the absence of zinc or in the presence of either phosphoramidon or bestatin, the network was much less regular (arrows indicate places where discontinuities exist in the network), the number of plasmatocytes per node varied considerably, and plasmatocytes were less aligned. At any time viewed (data not shown), networks were less organized in the absence of zinc or in the presence of phosphoramidon or bestatin.

Discussion

Our observations and measurements indicated that *M. sexta* plasmatocyte mean length in the presence of zinc (and absence of inhibitors) was significantly greater than in zinc's absence. As expected given our previous work involving calcium (Willott *et al.*, 2002) and the fact that EGTA would chelate both calcium and zinc, spread plasmatocytes were considerably shorter when EGTA was present compared to when it was absent (data not shown). The presence of zinc plus either of the two zinc-protease inhibitors, phosphoramidon and bestatin, resulted in average lengths of spread plasmatocytes similar to that seen in the absence of zinc. This suggests that at least one zinc protease is responsible for—or

contributes to—plasmatocyte spreading. The distribution of plasmatocyte sizes differed between the protease inhibitor treatments (as shown in the scatterplot, Figure 2), from which we infer that they inhibited different proteases, and hence, that at least two different zinc proteases are involved. This is consistent with the specificities of these two inhibitors. In general, at standard concentrations, phosphoramidon and bestatin inhibit different types of metalloproteases(Barrett *et al.*, 1998).

That the presence of zinc affected the behavior of plasmatocytes *in vitro* should not be surprising given zinc's many roles in mammalian immunity (for review, see Wellinghausen and Rink, 1998). At the molecular level, zinc affects: (1) adhesion of leukocytes to endothelial cells to facilitate movement of leukocytes into injured or inflamed tissue (Chavakis *et al.*, 1999); (2) receptor-based interaction of a natural killer cell or cytotoxic T lymphocyte with a potential target cell (Rajagopalan *et al.*, 1995); (3) protein-kinase C (PKC) activity (Csermely *et al.*, 1988) required for inactivation of IκB (Ghosh and Baltimore, 1990) and hence for activation and translocation of NF-κB (Shirikawa and Mizel, 1989)

Table 2. Effect of zinc, phosphoramidon, and bestatin on plasmatocyte width

	Width (µm)[a]			
	0 µM Zn	100 µM Zn	Phosphoramidon[b]	Bestatin[b]
#1	8.81 ± 3.0 (4.69-16.3)	10.8 ± 3.9 (4.69-19.9)	******	******
#2	11.3 ± 3.5 (4.33-22.7)	11.6 ± 3.7 (5.05-19.9)	******	******
#3	13.7 ± 4.8 (5.42-27.4)	13.9 ± 4.6 (4.69-24.2)	12.2 ± 4.9 (4.33-23.8)	12.7 ± 4.9 (5.05-26.4)
#4	10.6 ± 3.1 (4.69-17.7)	11.3 ± 3.8 (4.69-22.4)	10.9 ± 3.8 (4.33-21.3)	10.8 ± 3.4 (5.42-19.1)
#5	******	13.3 ± 3.6 (7.94-20.6)	12.3 ± 3.7 (5.42-19.9)	11.7 ± 3.4 (5.42-19.1)
#6	******	10.65 ± 3.6 (3.97-22.7)	13.76 ± 6.0 (5.78-30.0)	13.67 ± 4.2 (5.78-26.0)

a) Mean ± SD. N e" 50 for each test treatment. Range given in brackets.
b) 100 µM zinc was also present.

0 µM zinc vs. 100 µM zinc: #1-4 (uneven dashed box)

0 µM zinc vs. 100 µM zinc	p =.042

100 µM zinc vs. bestatin or phosphoramidon: #3-6 (even dashed box)

Bestatin vs. 100 µM zinc	p = 0.92
Phosphoramidon vs. 100 µM zinc	p = 0.97
Phosphoramidon vs. bestatin	p = 0.95

All factors co-analyzed: #3 & #4 (solid line)

0 µM zinc vs. 100 µM zinc	p = 0.45
Bestatin vs. 100 µM zinc	p = 0.14
Bestatin vs. 0 µM zinc	p = 0.47
Phosphoramidon vs. 100 µM zinc	p = 0.09
Phosphoramidon vs. 0 µM zinc	p = 0.34
Phosphoramidon vs. bestatin	p = 0.82

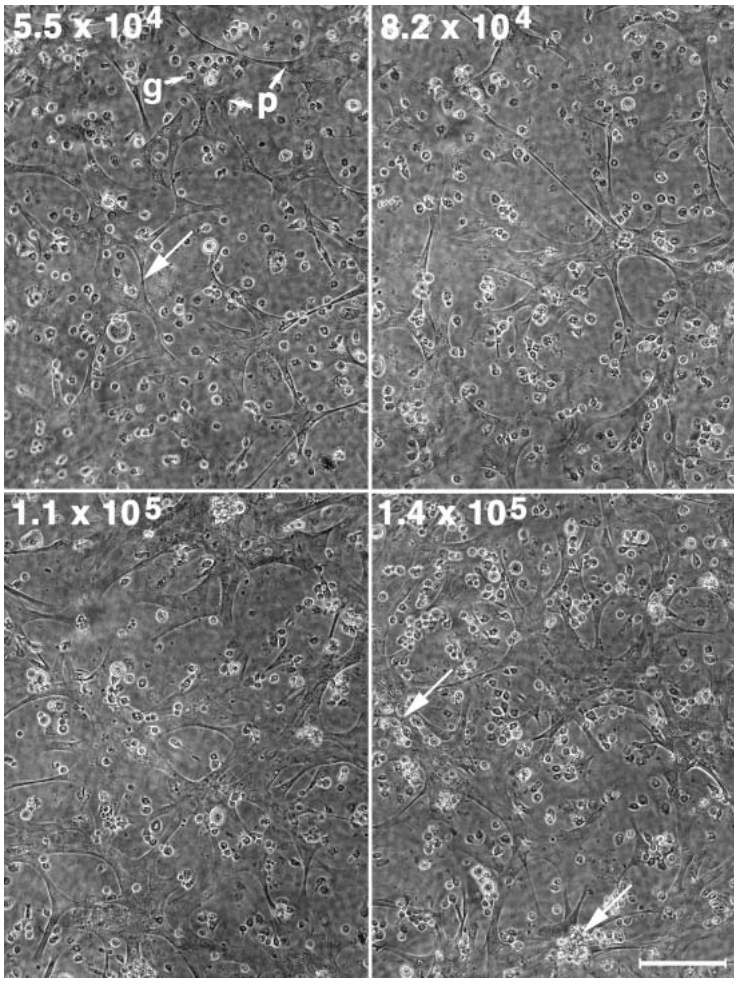

Figure 3. Phase contrast micrographs of hemocytes incubated 18 h on Nunclon∆ 96-well tissue culture plates. (a) 5.5 x 10^4 cells/well. (b) 8.7 x 10^4 cell/well. (c) 1.1 x 10^5 cell/well. (d) 1.4 x 10^5 cell/well. P indicates plasmatocytes; g indicates granular cell. At the lowest concentration, plasmatocytes connected (arrow points to two connected plasmatocytes) but failed to form an elaborate network; granular cells remained dispersed apparently randomly. At the highest concentration, plasmatocytes were primarily present in distinct nodes often with granular cells on and around them (arrows point to nodes with accompanying granular cells). Bar = 100 µm.

which leads to (4) DNA-binding of the transcription factor NF-κB (Zabel *et al.*, 1991) (Yang *et al.*, 1995) which stimulates gene expression of proteins of the innate immune response. Presumably comparable sites would be affected in the insect immune response given the remarkable similarities of certain insect proteins with mammalian NF-κB and IκB and other mammalian immune-related proteins (Kopp and Medzhitov, 1999; Hoffmann *et al.*, 1996).

Our work, in conjunction with reasonable parallels regarding the need for zinc in mammalian immunity, strongly suggests that *in vitro* assays on hemocyte function should include zinc in the medium since otherwise zinc-dependent processes may be overlooked. The concentration of zinc required may need to be determined for each species, since concentration of zinc in *M. sexta* hemolymph at approximately 100 µM, was notably higher than that of human serum, at 13-30 µM (Goyer, 1996 p.721) or monkeys, at 12 µM (Kaneko *et al.*, 1997 p. 899).

Proteases are crucial to any immune response studied to date. Insect serine proteases and their inhibitors have been relatively

well studied (some relevant papers include Kanost and Jiang, 1996; Jiang *et al.*, 1999; Kanost, 1999; Yamamoto *et al.*, 1999b; Gorman and Paskewitz, 2001). In contrast, only recently has exploration begun on the role of zinc-requiring metalloproteases in insect immunity.

Metalloproteases have been organized into clans and families based on sequence and structural similarities, the type and number of metal ion required, and which amino acids complex with the metal ion (Barrett *et al.*, 1998). Phosphoramidon, a natural metabolite produced by *Streptomyces tomashiensis*, inhibits primarily a particular endoprotease clan—Clan MA. Thermolysin, discussed above, belongs to Clan MA family M4 that so far contains only proteins of bacterial origin (Barrett *et al.*, 1998 p. 1033). Thermolysin structure is known to 1.6 Å (Holmes and Matthews, 1982); it has one zinc and four calcium binding sites, and, as discussed above, can cleave *G. mellonella* hemolymph proteins and thereby generate small peptides that induce expression of *G. mellonella* immune-related proteins. Another member of this clan, but from family M13 is the mammalian neutral endopeptidase 24.11, NEP (also called neprolysin) (for review, see Turner *et al.*, 2001). NEP metabolizes several proinflammatory peptides; for example, it degrades interleukin 1 (Pierart *et al.*, 1988). It shows transient expression in certain blood cell precursor cells and in mature leukocytes under some conditions (Turner, 1998 p.1083). NEP-knock-out mice are highly sensitive to endotoxins (Lu *et al.*, 1995), suggesting NEP's metabolism of proinflammatory peptides is highly important. An NEP-like activity is present on mollusc hemocytes (Ottaviani and Caselgrandi, 1997) and, as noted above, the fat body of bacteria-injected *M. sexta* larvae expresses an mRNA encoding an NEP-like sequence. Yet another protein in Clan MA, but from family M1, is leukotriene A_4 hydrolase (for review, see Haeggström, 1998). Metabolites of arachidonic acid, the leukotrienes, are lipid-based mediators of inflammation. Leukotriene A_4 hydrolase modifies leukotriene A_4 to the potent chemoattractant leukotriene B_4, but in the process destroys its own catalytic ability. In addition it cleaves several peptides, though which is the key peptide in vivo is not known. Interestingly, leukotriene A_4 hydrolase is one of the enzymes in clan MA that is inhibited by bestatin (Orning *et al.*, 1991).

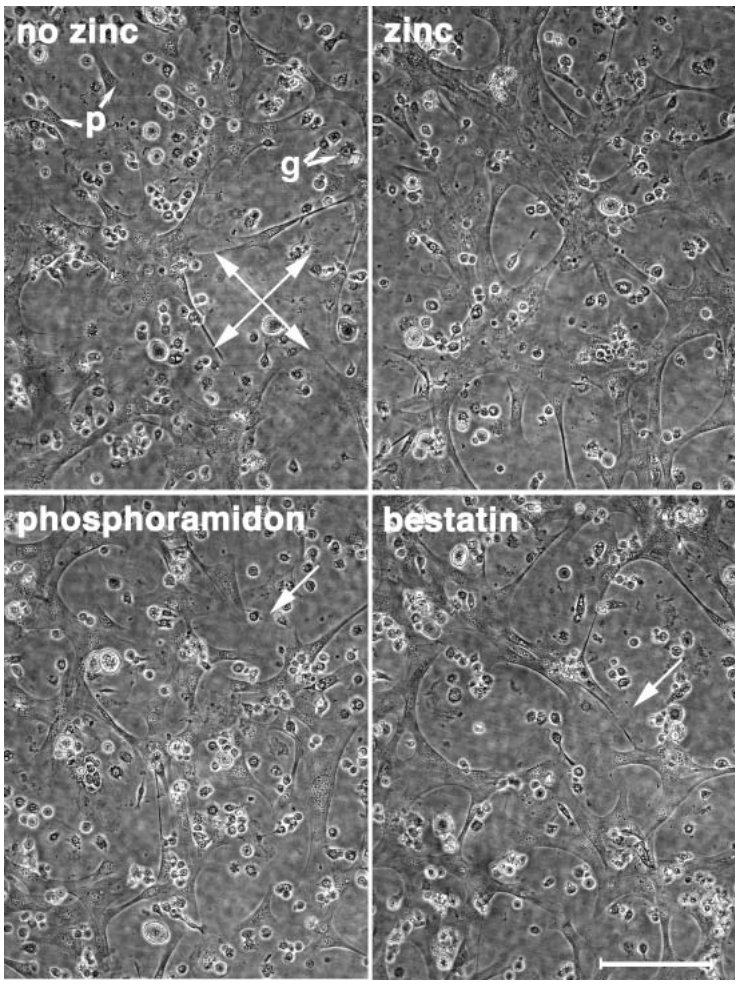

Figure 4. Phase contrast micrographs of hemocytes incubated 18 h on Nunclon∆ 96-well tissue culture plates. 1.1 x 10^5 hemocytes/well. P indicates plasmatocytes; g indicates granular cell. (a) 0 µM zinc. (b) 100 µM zinc. (c) 2.5 µM phosphoramidon and 100 µM zinc. (d) 1 µM bestatin and 100 µM zinc. Note the greater order (more consistent number of plasmatocytes per node; more consistent distance between nodes) in the network formed in the presence of zinc than in the absence of zinc or in the presence of either phosphoramidon or bestatin. Arrows in images for no-zinc, phosphoramidon, and bestatin treatments point to areas where connections would be expected if the network were regularly organized. Bar = 100 µm.

Bestatin, in contrast, is sometimes referred to as an aminopeptidase-specific inhibitor (Yasuhara, 1998), and inhibits proteases of several different clans, although in general it does not inhibit proteins of clan MA, unless used at high doses. One example of a metalloprotease involved in the mammalian immune response and inhibited by bestatin is an enzyme that activates antibacterial proteins stored in granules of immune cells (Lindmark *et al.*, 1999).

Another class of zinc-dependent proteases is the ADAM metalloproteinases, which are Clan B metalloproteases (for review, see Wolfsberg and White, 1998). These proteinases have both a disintegrin domain, which can interact with integrins, and a metalloprotease domain; several are regulated by proteolytic processing. More than five different ADAM family proteins are present in macrophages, key scavenging cells in mammalian innate immunity (Yamamoto *et al.*, 1999a).

An exogenous metalloprotease (thermolysin) acting on hemolymph proteins can produce peptide fragments capable of inducing production of antibacterial proteins in *G. mellonella* (Griesch *et al.*, 2000). Our work does not conflict with this result, rather our work suggests the possibility that *endogenous* metalloproteases play a role in the immune response in invertebrates. Support for this hypothesis include: (a) In the ascidian *Halocythia roretzi*, lipopolysaccharide induces release of a metalloprotease from hemocytes (Azumi *et al.*, 1991); (b) Following immune challenge of insect larvae, there is higher expression of sequences with similarity to zinc-dependent proteases, including one protein with similarity to human NEP 24.11 which is known to be involved in the immune response. Hence, an inducible metalloprotease inhibitor might have multiple roles: to help eradicate any exogenous threat persisting longer than a few hours and to participate in turning off an endogenous protease whose activity is no longer needed.

Cell-concentration clearly affected network formation, similar to what we reported in our study of calcium and hemocyte function (Willott *et al.*, 2002). At high concentrations of cells and with sufficient time, a network formed regardless of the presence of zinc or phosphoramidon or bestatin at the inhibitor concentrations tested. At lower cell concentrations, organized plasmatocyte-

plasmatocyte connections, and then networks, took longer to form. The inhibitors did not merely slow formation of a network: the structure of the networks differed, although sometimes subtly. In the presence of either inhibitor, the number of cells per node was much more variable than in the absence of the inhibitors, so the overall network was less uniform in appearance and spread plasmatocytes could be absent from relatively large areas, while adjacent areas contained giant nodes with, on average, many more cells than would be typically found in the absence of inhibitors. When shorter times of exposure to inhibitors were examined, a difference in the orientation of plasmatocytes with regard to each other could be seen—consistent with them not receiving or responding to a particular stimulus released from nearby plasmatocytes. It is reasonable to expect that multiple signals, including some redundant ones, are being sent and received, and that zinc and the zinc-protease inhibitors affect only a subset of these signals.

Zinc's affect on plasmatocytes could be direct, or it could act by influencing a signal from other hemocyte types. One of the anonymous reviewers of this manuscript noted that his or her group tested the effect of zinc on isolated *G. mellonella* plasmatocytes and saw a response similar to ours, suggesting that the effect is direct.

Proteins affecting plasmatocyte spreading have been identified from several lepidopteran species (for discussion, see Willott *et al.*, 2002). One such peptide, plasmatocyte spreading peptide or PSP, enhances plasmatocyte spreading (Strand and Clark, 1999). This peptide is made as a precursor that is cleaved to generate the active form (Clark *et al.*, 1998). The enzyme that cleaves it has not been identified. Spread plasmatocytes apparently release one or more factors to stimulate apoptosis of granular cells (Pech and Strand, 2000). It is possible that a zinc protease may be involved in (a) the cleavage of PSP, or (b) in the release of the factor that stimulates apoptosis. Metalloprotease-mediated cleavage of proteins is a theme in mammalian immunity (for one example, see Leca *et al.*, 1995). If a zinc-dependent protease is involved, it likely will be more readily discovered if zinc is routinely present or if zinc concentration is experimentally manipulated during assays or *in vitro* experiments involving insect hemocytes and the immune response.

Acknowledgements

We thank Mary Hernandez, and Dr. Michael A Wells (University of Arizona), for providing us with *M. sexta* eggs; the personnel at The University of Arizona Veterinary Diagnostic Clinic for atomic absorption spectroscopy measurements of zinc in *M. sexta* hemolymph and in Grace's medium; Dr. Yass Kobayashi for assistance with statistics; and Dr. Tina Trenczek, Giessen University, for many helpful discussions. This work was funded by NIH grant AI37722 (EW) and by The University of Arizona Small Grants to Faculty award (EW).

References

Azumi K, Yokosawa H, Ishii S-i. 1991. Lipopolysaccharide induces release of a metallo-protease from hemocytes of the ascidian, *Halocynthia roretzi*. *Developmental and Comparative Immunology* 15: 1-7.

Barrett AJ, Rawlings ND, Woessner JF, editors. 1998. *Handbook of proteolytic enzymes*. San Diego, Academic Press.

Bettencourt R, Gunne H, Gastinel L, Steiner H, Faye I. 1999. Implications of hemolin glycosylation and Ca^{2+}-binding on homophilic and cellular interactions. *European Journal of Biochemistry* 266: 964-976.

Bettencourt R, Lanz-Mendoza H, Lindquist KR, Faye I. 1997. Cell adhesion properties of hemolin, an insect immune protein in the Ig superfamily. *European Journal of Biochemistry* 250: 630-637.

Chavakis T, May AE, Preissner KT, Kanse SM. 1999. Molecular mechanisms of zinc-dependent leukocyte adhesion involving the urokinase receptor and β_2-integrins. *Blood* 93(9): 2976-2983.

Clark KD, Witherell A, Strand MR. 1998. Plasmatocyte Spreading Peptide is encoded by an mRNA differentially expressed in tissues of the moth *Pseudoplusia includens*. *Biochemical and Biopyhsical Research Communications* 250: 479-485.

Csermely P, Szamel M, Resch K, Somogyi J. 1988. Zinc can increase the activity of protein kinase C and contributes to its binding to plasma membranes in T lymphocytes. *Journal of Biological Chemistry* 263(14): 6487-6490.

Drif L, Brehélin M. 1994. Purification and characterization of an agglutinin from the hemolymph of *Locusta migratoria* (Orthoptera). *Insect Biochemistry and Molecular Biology* 24(3): 283-289.

Fernando-Warnakulasuriya GJP, Tsuchida K, Wells MA. 1988. Effect of dietary lipid content on lipid transport and storage during larval development of *Manduca sexta*. *Insect Biochemistry* 18: 211-214.

Ghosh S, Baltimore D. 1990. Activation *in vitro* of NF-κB by phosphorylation of its inhibitor IκB. *Nature* 344: 678-682.

Gillespie JP, Kanost MR, Trenczek T. 1997. Biological mediators of insect immunity. *Annual Review of Entomology* 42: 611-643.

Gorman MJ, Paskewitz SM. 2001. Serine proteases as mediators of mosquito immune responses. *Insect Biochemistry and Molecular Biology* 31(3): 257-262.

Goyer RA. 1996. Toxic effects of metals. In: Klaassen CD, editor. *Caserett and Doull's Toxicology: the basic science of poisons*. Fifth edition. pp. 691-736. New York, McGraw-Hill.

Griesch J, Vilcinskas A. 1998. Proteases released by entomopathogenic fungi impair phagocytic activity, attachment and spreading of plasmatocytes isolated from haemolymph of the greater wax moth *Galleria mellonella*. *Biocontrol Science and Technology* 8: 517-531.

Griesch J, Wedde M, Vilcinskas A. 2000. Recognition and regulation of metalloproteinase activity in the haemolymph of *Galleria mellonella:* a new pathway mediating induction of humoral immune responses. *Insect Biochemistry and Molecular Biology* 30: 461-472.

Haeggström JZ. 1998. Leukotriene A_4 hydrolase. In: Barrett

AJ, Rawlings ND, Woessner JF, editors. *Handbook of Proteolytic Enzymes* pp. 1022-1025. San Diego, Academic Press.

Hoffmann, JA, Reichhart, J-M, Hetru, C. 1996. Innate immunity in higher insects. *Current Opinion in Immunology* 8: 8-13.

Holmes MA, Matthews BW. 1982. Structure of thermolysin refined at 1.6 Å resolution. *Journal of Molecular Biology* 160: 623-639.

Jiang H, Wang Y, Kanost MR. 1999. Four serine proteinases expressed in *Manduca sexta* haemocytes. *Insect Molecular Biology* 8(1): 39-53.

Kaneko JJ, Harvey JW, Bruss ML, editors. 1997. *Clinical biochemistry of domestic animals*. Fifth edition. San Diego, Academic Press.

Kanost MR. 1999. Serine proteinase inhibitors in arthropod immunity. *Developmental and Comparative Immunology* 23: 291-301.

Kanost MR, Jiang H. 1996. Proteinase inhibitors in invertebrate immunity. In: Soderhall K, Iwanaga S, Vasta GR, editors. *New Directions in Invertebrate Immunology* pp. 155-173. Fair Haven NJ, SOS Publications.

Kopp, EB, Medzhitov, R. 1999. The Toll-receptor family and control of innate immunity. *Current Opinion in Immunology* 11: 13-18.

Leca G, Mansur SE, Bensussan A. 1995. Expression of VCAM-1 (CD106) by a subset of TCRγδ-bearing lymphocyte clones: involvement of a metalloprotease in the specific hydrolytic release of the soluble isoform. *Journal of Immunology* 154: 1069-1077.

Lindmark A, Garwicz D, Rasmussen PB, Flodgaard H, Gullberg U. 1999. Characterization of the biosynthesis, processing, and sorting of human HBP/CAP37/azurocidin. *Journal of Leukocyte Biology* 66: 634-643.

Lu B, Gerard NP, Kolakowski LF, Jr., Bozza M, Zurakowski D, Finco O, Carroll MC, Gerard C. 1995. Neutral endopeptidase modulation of septic shock. *Journal of Experimental Medicine* 181: 2271-2275.

Mandato CA, Diehl-Jones WL, Downer RGH. 1996. Insect hemocyte adhesion *in vitro*: inhibition by apolipophorin I and an artificial substrate. *Journal of Insect Physiology* 42(2): 143-148.

Orning L, Krivi G, Fitzpatrick FA. 1991. Leukotriene A_4 hydrolase: inhibition by bestatin and intrinsic aminopeptidase activity establish its functional resemblance to metallohydrolase enzymes. *Journal of Biological Chemistry* 266(3): 1375-1378.

Ottaviani E, Caselgrandi E. 1997. Neutral endopeptidase-24.11 (NEP)-like activity in Molluscan hemocytes. *Peptides* 18(8): 1107-1110.

Pech LL, Strand MR. 2000. Plasmatocytes from the moth *Pseudoplusia includens* induce apoptosis of granular cells. *Journal of Insect Physiology* 46: 1565-1573.

Pierart ME, Najdovski T, Appelboom TE, Deschodt-Lanckman MM. 1988. Effect of human endopeptidase 24.11 ("Enkephalinase") on IL-1 induced thymocyte proliferation activity. *Journal of Immunology* 140(11): 3808-3811.

Rajagopalan S, Winter CC, Wagtmann N, Long EO. 1995. The Ig-related killer cell inhibitory receptor binds zinc and requires zinc for recognition of HLA-C on target cells. *Journal of Immunology* 155: 4143-4146.

Ratcliffe NA. 1993. Cellular defense responses of insects: unresolved problems. In: Beckage NE, Thompson SN, Federici BA, editors. *Parasites and pathogens of Insects*Vol 1. Parasites. pp. 267-304. San Diego, Academic Press.

Shin SW, Park S-S, Park D-S, Kim MG, Kim SC, Brey PT, Park H-Y. 1998. Isolation and characterization of immune-related genes from the fall webworm, *Hyphantria cunea*, using PCR-based differential display and subtractive cloning. *Insect Biochemistry and Molecular Biology* 28: 827-837.

Shirikawa F, Mizel SB. 1989. *In vitro* activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. *Molecular and Cellular Biology* 9(6): 2424-2430.

Strand MR, Clark KD. 1999. Plasmatocyte spreading peptide induces spreading of plasmatocytes but represses spreading of granulocytes. *Archives of Insect Biochemistry and Physiology* 42: 213-223.

Strand MR, Pech LL. 1995. Immunological basis for compatibility in parasitoid-host relationships. *Annual Review of Entomology* 40: 31-56.

Tojo S, Naganuma F, Arakawa K, Yokoo S. 2000. Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. *Journal of Insect Physiology* 46: 1129-1135.

Trenczek T. 1998. Endogenous defense mechanisms of insects. *Zoology—Jena* 101(4): 298-315.

Turner AJ. 1998. Neprilysin. In: Barrett AJ, Rawlings ND, Woessner JF, editors. *Handbook of proteolytic enzymes* pp. 1080-1085. San Diego, Academic Press.

Turner AJ, Isaac RE, Coates D. 2001. The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. *Bioessays* 23(3): 261-269.

Wedde M, Weise C, Kopacek P, Franke P, Vilcinskas A. 1998. Purification and characterization of an inducible metalloprotease inhibitor from the hemolymph of greater wax moth larvae, *Galleria mellonella*. *European Journal of Biochemistry* 255: 535-543.

Wellinghausen N, Rink L. 1998. The significance of zinc for leukocyte biology. *Journal of Leukocyte Biology* 64: 571-577.

Willott E, Hallberg CA, Tran HQ. 2002. The influence of calcium on *Manduca sexta* plasmatocyte spreading and network formation. *Archives of Insect Biochemistry and Physiology* in press.

Willott E, Trenczek T, Thrower LW, Kanost MR. 1994. Immunochemical identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte types in *Manduca sexta*. *European Journal of Cell Biology* 65: 417-423.

Wolfsberg TG, White JM. 1998. ADAM metalloproteases. In:

Barrett AJ, Rawlings ND, Woessner JF, editors. *Handbook of proteolytic enzymes* pp. 1310-1313. San Diego, Academic Press.

Yamamoto S, Higuchi Y, Yoshiyama K, Shimizu E, Kataoka M, Hijiya N, Matsuura K. 1999a. ADAM family proteins in the immune system. *Immunology Today* 20(6): 278-284.

Yamamoto Y, Watabe S, Kageyama T, Takahashi SY. 1999b. Purification and characterization of *Bombyx* cysteine proteinase specific inhibitors from the hemolymph of *Bombyx mori*. *Archives of Insect Biochemistry and Physiology* 41: 119-129.

Yang J-P, Merin JP, Nakano T, Kato T, Kitade Y, Okamoto T. 1995. Inhibition of the DNA-binding activity of NF-κB by gold compounds *in vitro*. *FEBS Letters* 361: 89-96.

Yasuhara T. 1998. Aminopeptidase Y. In: Barrett AJ, Rawlings ND, Woessner JF, editors. *Handbook of proteolytic enzymes* pp. 1429-1430. San Diego, Academic Press.

Zabel U, Schreck R, Baeuerle PA. 1991. DNA binding of purified transcription factor NF-κB: affinity, specificity, Zn^{2+} dependence, and differential half-site recognition. *Journal of Biological Chemistry* 266(1): 252-260.