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The immune properties of *Manduca sexta* transferrin

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Abstract

Transferrins are secreted proteins that bind iron. The well-studied transferrins are mammalian serum transferrin, which is involved in iron transport, and mammalian lactoferrin, which functions as an immune protein. Lactoferrin and lactoferrin-derived peptides have bactericidal activity, and the iron-free form of lactoferrin has bacteriostatic activity due to its ability to sequester iron. Insect transferrin is similar in sequence to both serum transferrin and lactoferrin, and its functions are not well-characterized; however, many studies of insect transferrin indicate that it has some type of immune function. The goal of this study was to determine the specific immune functions of transferrin from *Manduca sexta* (tobacco hornworm). We verified that transferrin expression is upregulated in response to infection in *M. sexta* larvae and determined that the concentration of transferrin in hemolymph increases from 2 μ M to 10 μ M following an immune challenge. It is also present in molting fluid and prepupal midgut fluid, two extracellular fluids with immune capabilities. No immune-induced proteolytic cleavage of transferrin in hemolymph was observed; therefore, *M. sexta* transferrin does not appear to be a source of antimicrobial peptides. Unlike iron-saturated lactoferrin, iron-saturated transferrin had no detectable antibacterial activity. In contrast, 1 μ M iron-free transferrin inhibited bacterial growth, and this inhibition was blocked by supplementing the culture medium with 1 μ M iron. Our results suggest that *M. sexta* transferrin does not have bactericidal activity, but that it does have a bacteriostatic function that depends on its iron sequestering ability. This study supports the hypothesis that insect transferrin participates in an iron withholding strategy to protect insects from infectious bacteria.

Graphical abstract

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Keywords

Antimicrobial; Hemolymph; Innate immunity; Insect; Iron withholding; Transferrin

1. Introduction

Transferrins are secreted proteins that have a high affinity for iron (Lambert, 2012). The two best-understood transferrin family members are mammalian serum transferrin and mammalian lactoferrin (Lambert, 2012). Both proteins are made up of two lobes, and each lobe binds one ferric ion (Mizutani et al., 2012). Serum transferrin transports iron in the blood, while lactoferrin is an immune protein in extracellular fluids (Farnaud and Evans, 2003; Gkouvatsos et al., 2012).

Lactoferrin has bactericidal and bacteriostatic activity (Jenssen and Hancock, 2009). Iron-saturated (holo) and iron-depleted (apo) lactoferrin can kill bacteria in two ways: by interacting with the bacterial surface through a cationic patch on the lactoferrin surface, or by releasing antimicrobial peptides after undergoing proteolytic cleavage (Farnaud and Evans, 2003; Jenssen and Hancock, 2009). Apo-lactoferrin is bacteriostatic because it chelates iron and, thus, limits the amount of iron available to support bacterial growth (Farnaud and Evans, 2003; Jenssen and Hancock, 2009; Orsi, 2004).

Despite many excellent studies of insect transferrins, their specific functions are still poorly understood (Geiser and Winzerling, 2012). Insect transferrin sequences are approximately 30% identical to mammalian serum transferrin and lactoferrin sequences. Limited evidence suggests that transferrin may be involved in iron transport in insects (Huebers et al., 1988; Kurama et al., 1995), while many studies indicate that insect transferrin has some type of immune function (Geiser and Winzerling, 2012). Transferrin is present in hemolymph and has been detected in other extracellular fluids that contain immune proteins, including molting fluid from *Bombyx mori*, saliva from *Nephotettix cincticeps*, and seminal fluid from *Teleogryllus oceanicus* and *Melanoplus sanguinipes* (Bonilla et al., 2015; Geiser and Winzerling, 2012; Hattori et al., 2015; Qu et al., 2014; Simmons et al., 2013; J. Zhang et al., 2014). Transferrin is transcriptionally upregulated in response to infection in many species of insects, including representatives from Diptera, Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera (Geiser and Winzerling, 2012). Transferrin is downregulated in *Manduca sexta* parasitized by *Cotesia congregata*, and in *Glossinia morsitans* infected with trypanosomes, suggesting that parasitoids and parasites may benefit from suppressing transferrin expression (Chevignon et al., 2015; Lehane et al., 2008). Not much is known about immune-induced changes in the concentration of transferrin in hemolymph; however, fungal infection increased transferrin concentration in *Drosophila melanogaster* and *Protaetia brevitarsis*, and exposure to bacteria resulted in increased secretion of transferrin by cultured *Aedes aegypti* and *Aedes albopictus* cells (Kim et al., 2008; Levy et al., 2004; Yoshiga et al., 1997). Proteolytic cleavage products of insect transferrin have been observed, for example, in the hemolymph of *D. melanogaster* infected with a fungus (Levy et al., 2004); however, the functional significance of these polypeptides is unknown, and they are too large to be typical antimicrobial peptides. Two studies have identified immune-related

phenotypes resulting from RNAi-mediated knock down of transferrin: increased prevalence of trypanosome infections in *G. morsitans*, and increased mortality of infected *Plutella xylostella* (Kim and Kim, 2010; Lehane et al., 2008). Finally, transferrin from *Sarcophaga bullata* and *Bombyx mori* were shown to have antibacterial activity, although the transferrin used in these studies had an undetermined ratio of apo to holo forms, and, thus, it is unknown whether bacterial growth was inhibited by direct bactericidal activity or by iron chelation (Ciencialová et al., 2008; Yun et al., 2009). Clearly, insect transferrin must have at least one role in immunity, but its specific functions are unknown.

The aim of this study was to determine the immune functions of insect transferrin. We decided to study transferrin from *Manduca sexta* (tobacco hornworm) because the large size of this insect facilitates the collection of hemolymph and because previous studies of iron metabolism and immunity in *M. sexta* provide a good framework for this project (Bartfeld and Law, 1990; Cao et al., 2015; Gunaratna and Jiang, 2013; Huebers et al., 1988; Kanost et al., 2004; S. Zhang et al., 2014). RT-PCR was performed to detect immune-induced changes in transferrin transcript abundance, and immunoblot analysis was used to estimate the concentration of transferrin in extracellular fluids and to look for evidence of immune-induced proteolytic cleavage. In addition, we tested apo and holo forms of *M. sexta* transferrin for antibacterial activity. We found that *M. sexta* transferrin was present at a high concentration in extracellular fluids, that transferrin transcript abundance and concentration in hemolymph was increased in response to infection, and that transferrin's antimicrobial activity depended on its ability to sequester iron.

2. Materials and Methods

2.1. Insect culture

M. sexta were cultured at 26°C on a wheat germ-based artificial diet, with a photoperiod of 16 h of light and 8 h of darkness.

2.2. Collection of extracellular fluids

To collect plasma, hemolymph from two-day-old fifth instar larvae was centrifuged at $10,000 \times g$ for 5 min at 4°C to remove hemocytes, and the supernatant (plasma) was retained. Samples intended for immunoblot analysis were immediately heated with SDS sample buffer to decrease non-specific proteolytic degradation. Molting fluid was collected from a pharate adult by pricking the base of the proboscis with a needle and collecting the fluid that seeped out with a glass microcapillary tube. The fluid was immediately heated in SDS sample buffer. Midgut fluid was collected from a pharate pupa, which was chilled on ice, dissected along the dorsal midline, and rinsed several times with phosphate buffered saline. The midgut was dissected from the insect, transferred to a clean dish, rinsed three times with phosphate buffered saline, and blotted to remove excess buffer. The midgut was then cut midway along its length, and midgut contents were collected with a pipet and centrifuged at $10,000 \times g$ for 2 min. The supernatant was immediately heated with SDS sample buffer.

2.2. RNA isolation and RT-PCR

One-day-old fifth instar larvae were injected with 100 µl sterile 0.85% NaCl or 100 µl of 1 µg/µl *Micrococcus luteus* (American Type Culture Collection (ATCC) # 4698 from Sigma), and fat body samples were collected at 3, 6 and 24 h post injection (n = 3). (Note that we routinely use this method of inducing immune responses in *M. sexta* larvae, e.g., (Zhu et al., 2003).) Total RNA was isolated with the Tri reagent method (Sigma), and genomic DNA was removed with DNase I. The quality of DNase-treated total RNA was verified with a Bioanalyzer (Agilent). Pools of cDNA were synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) with oligo(dT) primers. Real-time PCR reactions were monitored on a CFX96 (Bio-Rad) by means of SYBR Green dye (Bio-Rad). Expression data were normalized to ribosomal protein subunit 3 (rpS3) expression (Jiang et al., 1996). The primer sequences for transferrin were 5' CCATTCGTCAGTAGTGTT 3' and 5' GTCAGGGCTATCAAAGTTA 3'; the sequences for rpS3 were 5' TCAGGCCGAGTCTTTGAGAT 3' and 5' AGCACTCCTTGCCTGAGAAG 3'. Relative changes in gene expression were calculated using delta delta Ct method (Livak and Schmittgen, 2001). Pairwise differences in expression were assessed by performing an unpaired *t* test. Statistical analyses were performed with GraphPad Prism software.

2.3. Production of polyclonal antiserum

Polyclonal antiserum was generated against residues A1-Y332 (amino-lobe) or L333-A663 (carboxyl-lobe) of the mature form of *M. sexta* transferrin. The corresponding partial cDNAs were amplified by PCR using amino-lobe primers, 5' GGATCCTTGCTAAATCTTCATACAAG 3' and 5' GCGGCCGCTAGTAGTCAATGGGTTT 3', or carboxyl-lobe primers, 5' GTCGACCTGAACAAAGCTAAC 3' and 5' GCGGCCGCTTAGGCGAGACCACATGA 3'. The cDNAs were digested with *NotI* and *Bam*HI (amino-lobe) or *NotI* and *Sa*II (carboxyl-lobe) and ligated into corresponding sites in pProEX HTc (ThermoFisher). Recombinant fusion proteins were expressed in the DH5α strain of *Escherichia coli* and were purified by nickel affinity chromatography under denaturing conditions. Purified protein (2.5 mg) was subjected to SDS-PAGE. Gels were stained with E-Zinc Reversible Stain Kit (Thermo Scientific), and the protein bands for each lobe were excised and sent to Pierce Biotechnology (Rockford, IL) for the production of polyclonal antiserum in rabbits.

2.4. Immunoblot analysis

Samples were subjected to SDS-PAGE followed by protein transfer to nitrocellulose. Immunodetection was done with a 1:2,000 dilution of polyclonal antiserum generated against either the amino-lobe or carboxyl-lobe of transferrin (see section 2.3), and then a 1:3,000 dilution of goat anti-rabbit IgG-AP conjugate secondary antibody (Bio-Rad) followed by color development via AP Conjugate Substrate Kit (Bio-Rad).

2.5. Determination of the concentration of transferrin in hemolymph

Plasma samples (0.1 µl, n = 3) from two-day-old fifth instar larvae and 10, 20, and 30 ng transferrin purified from hemolymph (see section 2.8) were subjected to SDS-PAGE followed by immunodetection with the anti-carboxyl-lobe antiserum. Images of bands were

captured and net intensity was determined using Carestream software. A standard curve was generated, and the concentration of transferrin was determined by interpolation with the use of GraphPad Prism software.

2.6. Analysis of the effect of inoculation on transferrin concentration in plasma

One-day-old fifth instar larvae were injected with 100 μ l sterile 0.85% NaCl or 100 μ l of 1 μ g/ μ l *M. luteus* (ATCC # 4698 from Sigma), and plasma was collected at 24 and 48 h (n = 7). Plasma samples (0.1 μ l) and 5, 10, 20, 40, 80, and 100 ng recombinant carboxyl-lobe (described in section 2.3) were subjected to SDS-PAGE followed by immunodetection with the anti-carboxyl-lobe antiserum. Images of bands were captured and net intensity was determined using Carestream software. A standard curve was generated, and an arbitrary measure of transferrin concentration was determined by interpolation with the use of GraphPad Prism software. (Because the standard curve for this experiment was generated from recombinant carboxyl-lobe standards rather than purified native transferrin, a relative measure of transferrin concentration was possible, but a true measure was not.) Pairwise differences in expression were assessed by performing an unpaired *t* test; Welch's correction was used when variances were significantly different. Statistical analyses were performed with GraphPad Prism software.

2.7. Analysis of the effect of inoculation on transferrin cleavage

To detect immune-induced cleavage products of transferrin, the immunoblots described in section 2.6 were inspected. Those blots were optimized for separation, transfer and retention of polypeptides ranging from approximately 20–100 kDa. The gels used were Bis-Tris NuPAGE gels run with MOPS buffer (Life Technologies), and transfer occurred at 10 volts for 1 h.

To detect smaller cleavage products, 0.5 μ l plasma samples (3, 6 and 24 h post inoculation, n = 3) were analyzed using conditions optimized for detecting peptides. Peptides and polypeptides were separated on 16% Tricine gels (Life Technologies), and transferred to 0.22 μ m nitrocellulose for 1 h at 6 volts. To confirm that peptides did not pass through the first nitrocellulose membrane, a backup (second) membrane was stained with amido black and inspected to verify that peptides were not detected. To confirm that peptide transfer was efficient, the gels were stained with Coomassie Blue R-250 after protein transfer. Immunodetection was performed using antiserum against the amino-lobe for one blot and against the carboxyl-lobe for a second blot.

A second method to detect smaller cleavage products involved heating plasma samples to precipitate heat unstable proteins so that larger samples could be evaluated by immunoblot analysis. (Because of the high concentration of storage proteins (e.g., hexamerins), 0.5 μ l is the maximum volume of untreated plasma that can be separated by the methods we used for SDS-PAGE.) Three biological replicates (50 μ l each) of plasma were pooled, heated at 80°C for 15 min, chilled on ice for 20 min, and then centrifuged at $16,875 \times g$ for 20 min at 4°C to pellet precipitated proteins. The supernatant was heated with 6 $\times$ SDS sample buffer, and 12.5 μ l supernatant was subjected to SDS-PAGE with the use of 4–12% Bis-Tris NuPAGE gels run with MES Buffer, and then transferred to nitrocellulose for 1 h at 6 volts. Four

replicate blots were analyzed by immunodetection using the amino-lobe antiserum, carboxyl-lobe antiserum, or one of the corresponding preimmune sera.

A third method to detect smaller cleavage products involved *in vitro* immune activation. Hemolymph collected from two-day-old fifth instar larvae was centrifuged at $10,000 \times g$ for 5 min at 4°C. Plasma (supernatant) was transferred to a fresh tube. *M. luteus* (ATCC # 4698 from Sigma) was added to plasma at a final concentration of 0.5 µg/µl, or an equivalent volume of sterile 0.85% NaCl was added to control plasma samples. Control and immune-activated plasma samples were incubated at room temperature for 30 min and then heated with SDS sample buffer. Samples (0.5 µl) were subjected to SDS-PAGE on 16% Tricine gels, and peptides and proteins were transferred to nitrocellulose for 1 h at 6 volts. Immunodetection was performed using antiserum against the amino-lobe for one blot and against the carboxyl-lobe for a second blot.

2.8. Purification of *M. sexta* transferrin

Transferrin was purified from larval hemolymph using a previously published method (Huebers et al., 1988) with some significant modifications. Hemolymph was collected from two-day-old fifth instar larvae into saturated ammonium sulfate at 4°C. Ammonium sulfate was adjusted to 55% saturation, and precipitated proteins were pelleted by centrifugation. The supernatant (which was judged by immunoblot analysis to contain approximately 75% of the transferrin) was dialyzed twice against 20 mM Tris, 20 mM NaCl, pH 8.3 (4°C). The dialyzed sample (360 ml) was applied to a DEAE Sephacel column (2.5 × 22 cm), and proteins were eluted with a linear gradient of 0–1 M NaCl in 20 mM Tris, pH 8.3 (4°C). Approximately 50% of the transferrin was in the unbound protein fraction. This fraction was concentrated to 4 ml with Amicon Ultracel centrifugal filters with a 30 kDa molecular weight cut-off and then applied to a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated in 20 mM Tris, 150 mM NaCl, pH 8. Fractions containing transferrin were pooled and dialyzed twice against 20 mM Tris, 10 mM NaCl, pH 8.3. This sample was applied to an UnoQ1 column (Bio-Rad) and eluted with a linear gradient of 10–500 mM NaCl in 20 mM Tris, pH 8.3. Fractions with pure transferrin were pooled and dialyzed against 40 mM potassium phosphate, 20 mM NaCl, pH 7. With this method, 6.5 mg transferrin was purified from 84 ml hemolymph.

2.9. Production of apo-transferrin and holo-transferrin

Purified transferrin was concentrated to 5 mg/ml and the buffer was exchanged to 20 mM potassium phosphate, 20 mM NaCl, pH 7, with the use of an Amicon Ultracel centrifugal filter with a 30 kDa molecular weight cut-off. One mg was saved as “transferrin purified from hemolymph”. This sample was a reddish yellow color. The remaining 5.5 mg sample was divided into two aliquots, one for the production of apo-transferrin and one for the production of holo-transferrin.

Apo-transferrin was made by dialyzing 2.25 mg purified transferrin against 1 liter 0.1 M sodium acetate, 10 mM EDTA, pH 5, for 8 hours and then against 1 liter of the same buffer for 17 h. To remove EDTA and exchange the buffer to one that would be suitable for

biological assays, the sample was dialyzed twice against 0.5 liter 20 mM potassium phosphate, 20 mM NaCl, pH 7. The apo-transferrin was colorless.

Holo-transferrin was made by a previously published method (Battin et al., 2009). Briefly, sodium bicarbonate was added to 2.25 mg purified transferrin such that the final concentration was 20 mM, and an absorbance spectrum (from 250–900 nm) was recorded. To saturate the transferrin with iron, 0.1 molar equivalent of ferric-nitrilotriacetic acid was added, binding was allowed to occur for 5 min at room temperature, and the effect on absorbance was determined by recording an absorbance spectrum. No change in absorbance at 450 nm was observed. This process was repeated once more, and, again, no increase in absorbance was observed. Unbound iron was removed with a Sephadex G-25 column and dialyzed twice against 500 ml 20 mM potassium phosphate, 20 mM NaCl, pH 7. The holo-transferrin was a reddish yellow color. Note that we have used this method to generate holo-transferrin from bovine apo-transferrin and *M. sexta* apo-transferrin, and for those protein samples, absorbance at 450 nm increased after each addition of ferric-nitrilotriacetic acid until saturation was reached (data not shown); therefore, we conclude that the reason that we did not detect a change in absorbance when we added ferric-nitrilotriacetic acid to purified *M. sexta* transferrin was that it was already saturated with iron.

The concentrations of purified transferrin, apo-transferrin and holo-transferrin were adjusted to 50 μ M in 20 mM potassium phosphate, 20 mM NaCl, pH 7, and their integrity and concentration were verified by SDS-PAGE. Protein concentration was determined as described previously (Lang et al., 2012). An absorbance spectrum of each protein at 50 μ M was recorded.

2.10. Modeling surface electrostatics

The three-dimensional structure of *M. sexta* transferrin has not been determined; therefore, I-TASSER software (Roy et al., 2010; Zhang, 2008) was used to predict the structure of transferrin, and PyMOL (Schrödinger) was used to visualize the resulting homology model. To generate an electrostatic surface model of human lactoferrin (pdb: 1LFG) and *M. sexta* transferrin (homology model), the vacuum electrostatics calculation in PyMOL was used.

2.11. Effect of holo-transferrin on bacterial growth

E. coli (Coli Genetic Stock Center # 7636), *M. luteus* (ATCC # 700256), *Staphylococcus epidermidis* (ATCC # 14990), *Serratia marcescens* (ATCC # 13880) and *Enterococcus faecalis* (ATCC # 700802) were grown in nutrient broth (*M. luteus*, *S. epidermidis*, *S. marcescens*), LB-Miller (*E. coli*) or Todd Hewitt broth (*E. faecalis*). Cultures (200 μ l) were grown in sterile 96 well plates sealed with a Breathe-Easy gas permeable sealing membrane (Diversified Biotech) in a heated PowerWave XS microplate reader (BioTek) with constant shaking. Cultures were grown at 37°C, except for *S. marcescens*, which was grown at 30°C. Cultures were treated with either 2 μ M holo-transferrin or an equal volume of transferrin storage buffer (20 mM potassium phosphate, 20 mM NaCl, pH 7). OD₆₀₀ was measured every five min for 8 h. Three replicate cultures were analyzed per treatment. Growth curves were graphed in GraphPad Prism software.

2.12. Effect of apo-transferrin on *E. coli* growth

MOPS medium was made as described previously (Neidhardt et al., 1974) with the omission of ferrous sulfate. Wild-type *E. coli* (CGSC Strain # 7636) was grown at 37°C and 275 rpm in MOPS media with 5 µM ferrous sulfate and then overnight in MOPS medium supplemented with 1 µM ferrous sulfate. Bacteria from an overnight culture were washed and re-suspended to an OD₆₀₀ of 1.6 in MOPS medium without added iron, and 5 µl was used for each 200 µl final culture. Six treatments were used for this experiment. Each culture was grown in MOPS medium without added iron or MOPS medium supplemented with 1 µM ferrous sulfate, and each culture contained 1 µM apo-transferrin, 1 µM holo-transferrin, or an equal volume of transferrin storage buffer (20 mM potassium phosphate, 20 mM sodium chloride buffer, pH 7). Cultures were grown in sterile 96 well plates sealed with a Breathe-Easy gas permeable sealing membrane (Diversified Biotech) in a PowerWave XS microplate reader (BioTek) with constant shaking at 37°C. OD₆₀₀ was measured every five min for 8 h. Three replicate cultures were analyzed per treatment. Growth curves were graphed in GraphPad Prism software. In addition, changes in OD₆₀₀ (mean ± SD) over the course of the experiment were calculated. Differences in means were assessed by performing a one-way ANOVA followed by a Dunnett test.

Results

3.1. Transferrin is present in extracellular fluids at a high concentration

M. sexta transferrin is known to be present in hemolymph (Bartfeld and Law, 1990; Huebers et al., 1988). We were interested in whether transferrin is present in other extracellular fluids that contain immune-related proteins. Using immunoblot analysis, we found that transferrin is present in molting fluid and midgut fluid at a concentration that is similar to that of transferrin in hemolymph (Figure 1). To determine the concentration of transferrin in hemolymph, we made an immunoblot of plasma from fifth instar larvae and known amounts of purified transferrin (see section 3.4), and then used imaging and graphing software to generate a standard curve and determine the concentration. The concentration in hemolymph was calculated to be 178 ± 26 µg/ml, or approximately 2 µM.

3.2. Transferrin is upregulated in response to an immune challenge

A previous transcriptome study suggested that *M. sexta* transferrin is upregulated in fat body 24 h after an immune challenge (He et al., 2015). We used RT-PCR to determine when transferrin transcript abundance increases in response to inoculation with bacteria. We observed no difference at 3 and 6 h post-inoculation, but there was a three fold increase at 24 h (Figure 2A). A pilot experiment suggested that the concentration of transferrin in hemolymph was unchanged 3 and 6 h after inoculation but that the concentration was higher at 24 h (data not shown). An immunoblot analysis of plasma samples 24 and 48 h after inoculation demonstrated a four fold increase in the concentration of transferrin (Figures 2B and 3).

3.3. Transferrin does not appear to be cleaved in response to infection

Lactoferrin can be cleaved by proteolytic enzymes to release antimicrobial peptides (Farnaud and Evans, 2003), and cleavage products of fish transferrin (corresponding to the amino- and carboxyl-lobes) can activate macrophages (Stafford and Belosevic, 2003); therefore, we used several strategies to try to detect evidence of proteolytic cleavage of transferrin in hemolymph. We performed immunoblot analysis of 0.1 μ l plasma samples using conditions favoring the transfer and retention of polypeptides in the 20–80 kDa range, and we did not observe any evidence of proteolytic cleavage (Figure 3). We also performed immunoblot analysis of 0.5 μ l plasma samples using conditions that favor the transfer and retention of peptides but did not detect immuno-reactive peptides (data not shown). Because 0.5 μ l plasma contains the maximum amount of protein that could be separated by our SDS-PAGE system, we heated the samples to precipitate the highly abundant storage proteins and then performed immunoblot analysis of 12.5 μ l samples, but, again, we observed no immuno-reactive peptides (Figure S1A). Finally, we analyzed plasma samples that had been incubated with *M. luteus* for 30 min to initiate immune pathways, and no immuno-reactive peptides were detected (Figure S1B).

3.4. Generation of apo and holo forms of *M. sexta* transferrin

To purify *M. sexta* transferrin from hemolymph, we used ammonium sulfate precipitation followed by anion exchange chromatography, gel filtration, and high resolution ion exchange chromatography. From 84 ml larval hemolymph, we purified 6.5 mg transferrin (Figure 4A). We generated apo-transferrin by dialyzing purified transferrin against 10 mM EDTA in acetate buffer at pH 5 and then removed EDTA by dialysis against phosphate buffer (Figure 4A). We generated holo-transferrin by incubating purified transferrin with ferric-nitrilotriacetic acid in the presence of 20 mM sodium bicarbonate and then removed unbound iron with a desalting column and dialysis against phosphate buffer (Figure 4A). Each transferrin sample was adjusted to a concentration of 50 μ M in 20 mM potassium phosphate, 20 mM NaCl, pH 7. The holo-transferrin had a broad absorbance shoulder at approximately 450 nm, which is typical of ferric-transferrin (Patch and Carrano, 1981), whereas the apo-transferrin lacked this shoulder (Figure 4B). The absorbance spectrum of purified transferrin and holo-transferrin were essentially identical (Figure 4B). Because a significant fraction of transferrin in hemolymph is in the apo form (Huebers et al., 1988), we assume that our purification strategy favored holo-transferrin or that iron saturation of transferrin occurred during the purification process.

3.5. Holo-transferrin did not influence bacterial growth

Holo-lactoferrin and two purified insect transferrins of unknown iron-saturation status have antibacterial activity (Ciencialová et al., 2008; Jenssen and Hancock, 2009; Yun et al., 2009); therefore, we tested *M. sexta* holo-transferrin for an ability to inhibit bacterial growth. The bacteria tested included two Gram negative species (*E. coli* and *S. marcescens*) and three Gram positive (*M. luteus*, *S. epidermidis*, and *E. faecalis*). *S. marcescens* and *E. faecalis* are insect pathogens. The bacteria were grown in the presence or absence of 2 μ M holo-transferrin. This concentration was chosen because it is the concentration of transferrin in hemolymph. Growth was monitored for 8 h, and the effect of holo-transferrin was

evaluated. We observed no growth inhibition of any of the species of bacteria tested, and *M. luteus* and *S. epidermidis* actually grew better in the presence of holo-transferrin, suggesting that they were able to use holo-transferrin as a nutrient source (Figure 5 and Figure S2).

3.6. Transferrin does not appear to have a cationic surface patch

The bactericidal activity of lactoferrin is mediated by a cationic patch on the surface of the amino-lobe (Baker et al., 2002; Jenssen and Hancock, 2009). We were curious if *M. sexta* transferrin has a similar cationic patch. The structure of transferrin from *M. sexta* has not been determined; therefore, we generated a homology model using I-TASSER software (Roy et al., 2010; Zhang, 2008), and then used PyMOL to generate an electrostatic surface model. The entire electrostatic surface model was examined, and no large (more than a few amino acids) cationic surface patches were visible (Figure 6).

3.7. Apo-transferrin inhibits growth of *E. coli*

Vertebrate apo-transferrins are bacteriostatic because they sequester iron (Bezkorovainy, 1981); therefore, it seemed likely that insect apo-transferrin would also have this immune function. To test this hypothesis, we evaluated whether *M. sexta* apo-transferrin could inhibit growth of *E. coli*. To mimic iron availability in hemolymph, we grew *E. coli* in MOPS medium with no added iron such that the only iron present originated from the trace amounts in the chemicals and water used to make the medium. (Note that the concentration of non-protein-bound iron in *M. sexta* hemolymph is so low as to be undetectable (Adamo et al., 2007), a condition similar to that of human plasma, which was estimated to have a non-protein-bound iron concentration of approximately 10^{-18} M (Anderson and Vulpe, 2009; Drakesmith and Prentice, 2012).) For this experiment, we assumed that 50% of the transferrin in hemolymph is in the apo form, and, thus, that the concentration of apo-transferrin in hemolymph is approximately 1 μ M. *E. coli* were grown in the presence or absence of 1 μ M apo-transferrin. Growth was monitored for 8 h, and the effect of apo-transferrin was evaluated. We observed a significant decrease in the growth of bacteria in the presence of apo-transferrin (Figures 7 and 8A).

3.8. The growth-inhibiting activity of transferrin depends on its ability to sequester iron

We expected that the growth-inhibiting activity of apo-transferrin would be dependent on its ability to sequester iron. We used two methods to test this hypothesis. First, we grew *E. coli* in MOPS medium with no added iron, and, instead of adding apo-transferrin, we treated with 1 μ M holo-transferrin. Unlike apo-transferrin, treatment with holo-transferrin had no bacteriostatic activity (Figure 8A). In fact, the bacteria grew better in the presence of 1 μ M holo-transferrin, suggesting that *E. coli* can use *M. sexta* holo-transferrin as a nutrient source. Second, we grew *E. coli* in MOPS medium supplemented with 1 μ M ferrous sulfate and subjected them to one of three treatments: 1 μ M apo-transferrin, 1 μ M holo-transferrin, or an equivalent volume of buffer. (Note that *M. sexta* transferrin binds one ferric ion per molecule of protein (Bartfeld and Law, 1990; Huebers et al., 1988).) When the bacteria had access to 1 μ M iron in the medium, the presence of apo- or holo-transferrin had little or no effect (Figure 8B).

Discussion

The goal of this study was to determine the specific immune functions of *M. sexta* transferrin. We based our hypotheses mainly on the well-established immune functions of mammalian lactoferrin. We found no evidence that *M. sexta* transferrin has direct bactericidal activity, but our results indicate that insect transferrin has bacteriostatic activity that is dependent on its ability to chelate iron.

We found that *M. sexta* transferrin is present at a high concentration in molting fluid and midgut fluid, both of which are known to have immune properties and are unlikely to have a role in iron transport (Russell and Dunn, 1996; J. Zhang et al., 2014). Our findings are in agreement with other studies that detected transferrin in molting fluid, saliva and seminal fluid (Bonilla et al., 2015; Geiser and Winzerling, 2012; Hattori et al., 2015; Qu et al., 2014; Simmons et al., 2013; J. Zhang et al., 2014). We found that even healthy larvae had a high concentration of transferrin (~2 μ M), and the increase in concentration following inoculation was moderate and relatively slow (four fold by 24 h). Most insect antimicrobial proteins have little or no expression in healthy insects, are transcriptionally upregulated within a few hours in response to an immune challenge, and quickly increase in concentration to approximately 0.5–100 μ M in hemolymph in response to infection (Cociancich et al., 1994; Lemaitre and Hoffmann, 2007). *M. sexta* transferrin is similar to lactoferrin in that they both are present at high concentrations in extracellular fluids of healthy individuals; for example, the concentration of lactoferrin in milk and tears is approximately 20–60 μ M (Weinberg, 2007).

We used several strategies to test the hypothesis that transferrin undergoes immune-induced cleavage in hemolymph. We were interested in this possibility for three reasons: lactoferrin is cleaved by proteolytic enzymes to release antimicrobial peptides (Farnaud and Evans, 2003), fish transferrin that has been cleaved between the amino- and carboxyl-lobes acts as an activator of macrophages (Stafford and Belosevic, 2003), and *D. melanogaster* transferrin is cleaved into separate amino- and carboxyl-lobes in flies infected with the fungus *Beauveria bassiana* (Levy et al., 2004). In addition, proteolytic cleavage products of transferrin were detected in whole body and tissue homogenates from *Aedes aegypti* and *Glossina morsitans* (Guz et al., 2007; Harizanova et al., 2005). In our study, in which chilled hemolymph samples were quickly collected and processed, we did not detect any immune-induced cleavage products of transferrin; therefore, *M. sexta* transferrin does not appear to be a source of antimicrobial peptides or signaling molecules.

Lactoferrin has bactericidal activity that is independent of its ability to release antimicrobial peptides and chelate iron (Farnaud and Evans, 2003; Jenssen and Hancock, 2009). In addition, two insect transferrins that were not deliberately depleted of iron were shown to have antibacterial activity (Ciencialová et al., 2008; Yun et al., 2009). Because of these previous findings, we thought that *M. sexta* holo-transferrin may have bactericidal activity; however, in our experiments, 2 μ M holo-transferrin did not inhibit the growth of *E. coli*, *S. marcescens*, *M. luteus*, *S. epidermidis*, or *E. faecalis*. The transferrin purified from hemolymph of *Sarcophaga bullata* and used at concentrations similar to those of our study inhibited the growth of *Staphylococcus aureus* and *E. coli* (Ciencialová et al., 2008). The *S.*

bullata transferrin was purified by reverse phase chromatography using conditions that typically denature larger proteins such as transferrin; therefore, it is possible that iron was lost from the protein during the purification process and, thus, that the antimicrobial activity was due to iron chelation. Recombinant *Bombyx mori* transferrin used at 0.01 μ M inhibited the growth of *M. luteus*, *Bacillus subtilis*, *Enterococcus hirae*, *Pseudomonas aeruginosa*, and *E. coli*, but not *S. aureus* or *E. faecalis* (Yun et al., 2009). All of the cultures were grown in tryptic soy broth, which has an iron concentration of approximately 9 μ M (Ledala et al., 2014; Yun et al., 2009). The observation that recombinant *B. mori* transferrin had antibacterial activity in these experiments is surprising because the concentration of transferrin was much lower than that of natural concentrations of antimicrobial peptides and lactoferrin, and because the effect of iron chelation would have been minimal due to the much lower concentration of transferrin relative to that of iron in the medium (0.01 μ M vs 9 μ M). We cannot explain the discrepancy between the activity of *M. sexta* and *B. mori* transferrin (which are 86% identical in amino acid sequence), but perhaps the difference is due to the metal binding tag on the *B. mori* transferrin or a difference in experimental conditions. Given that no cationic surface patches were visible on the predicted surface of *M. sexta* transferrin, it is perhaps not surprising that *M. sexta* holo-transferrin had no detectable bactericidal activity.

It has been known for many years that vertebrate apo-transferrins are bacteriostatic because they sequester iron (Bezkorovainy, 1981). This immune mechanism was proposed for insect transferrin following the discovery that mosquito transferrin is an immune responsive protein (Yoshiga et al., 1997). Our study directly tested this hypothesis by comparing the effects of apo- and holo-transferrin on the growth of *E. coli*. We found that 1 μ M apo-transferrin inhibited bacterial growth, whereas the same concentration of holo-transferrin did not. In addition, we demonstrated that the growth-inhibiting activity of apo-transferrin was blocked by iron supplementation of the culture medium. Our results provide direct evidence that an insect apo-transferrin can interfere with bacterial growth by means of iron sequestration; therefore, our study supports the model that insect transferrin participates in an iron withholding strategy in hemolymph and other extracellular fluids to protect insects from infectious bacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATCC American Type Culture Collection

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Highlights

- *Manduca sexta* transferrin (Tf) is present in hemolymph, molting fluid and prepupal midgut fluid at a high concentration.
- Inoculation with bacteria increases MsTf transcript abundance in fat body and MsTf concentration in hemolymph 3–4 fold.
- Iron-saturated MsTf has no detectable antibacterial activity, whereas iron-free MsTf inhibits growth of bacteria.
- MsTf appears to participate in an iron withholding strategy to protect the insect from infectious bacteria.

Figure 1.

Immunoblot analysis of transferrin in extracellular fluids. The samples were 0.5 μ l plasma (P) from feeding stage fifth instar larvae, 0.5 μ l molting fluid (MF) from pharate adults, 0.5 μ l midgut fluid (GF) from pharate pupae, and 50 ng purified transferrin (Tf). The positions of molecular mass standards (in kDa) are shown to the left of the blot. Note that transferrin in plasma is detected as a curved, faster migrating band due to the high concentration of storage proteins that migrate just behind transferrin. Each extracellular fluid had a similar concentration of transferrin.

Figure 2.

Effect of immune challenge on the expression and concentration of transferrin. (A) RT-PCR was used to detect changes in mRNA abundance at 3, 6 and 24 h post-inoculation with bacteria. qPCR data were from three biological replicates. Means $\pm$ SD are shown. Differences in expression were assessed by performing an unpaired *t* test. An increase in mRNA abundance at 24 h post-inoculation was observed. (B) Immunoblot analysis was used to detect changes in transferrin concentration in plasma at 24 and 48 h post-inoculation with bacteria. Data were from seven biological replicates. Means $\pm$ SD are shown. Differences in expression were assessed by performing an unpaired *t* test with Welch's correction. An increase in transferrin concentration at 24 h and 48 h post-inoculation was observed. (**P* < 0.05, ****P* < 0.001) (Abbreviations: C, control; In, inoculated.)

Figure 3.

Immunoblot analysis to detect proteolytic cleavage of transferrin in response to immune challenge. Plasma samples (0.1 μ l) from saline-injected ($n = 7$) and bacteria-inoculated ($n = 7$) larvae, 24 h post-injection, were analyzed. The positions of molecular mass standards (in kDa) are shown to the left of the blot. No evidence of cleavage was detected.

Figure 4.

Analysis of purified forms of transferrin. (A) Transferrin purified from hemolymph (Tf), apo-transferrin (aTf), and holo-transferrin (hTf) were analyzed by reducing SDS-PAGE followed by Coomassie staining. Each lane contains 0.25 μ g transferrin. The positions of molecular mass standards (in kDa) are shown on the left. No contaminating proteins were detected. (B) An absorbance spectrum of each of the samples analyzed by SDS-PAGE is presented. The protein concentration was 50 μ M, and the path length was 0.5 cm.

Transferrin purified from the hemolymph is indicated by a green line, apo-transferrin by a

blue line, and holo-transferrin by a red line. Note that the absorbance spectrum of purified transferrin and holo-transferrin is essentially identical; therefore, only the red line is visible. A broad absorbance shoulder at approximately 450 nm indicates the presence of bound ferric ion.

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Figure 5.

Effect of holo-transferrin on the growth of *E. coli*. Bacteria were grown in LB medium with 2 μ M holo-transferrin (red) or an equivalent volume of buffer (gray). Data from three replicate cultures are shown. Error bars indicate standard deviation. Holo-transferrin had no effect on the growth of *E. coli*.

Figure 6.

Surface electrostatics of human lactoferrin and *M. sexta* transferrin. PyMOL was used to generate an electrostatic surface model for (A) a crystallographic structure of human lactoferrin (pdb: 1LFG), and (B) a homology model of *M. sexta* transferrin. The structures are shown in the same orientation (amino lobe on the left, and amino-terminus to the front). Blue indicates positive charge; red indicates negative charge. An amino-terminal cationic surface patch is clearly visible in the lactoferrin model. No similar cationic surface patches were detected for *M. sexta* transferrin.

Figure 7.

Effect of apo-transferrin on the growth of *E. coli*. Bacteria were grown in MOPS medium with no added iron and either 1 μ M apo-transferrin (blue) or an equivalent volume of buffer (gray). Data from three replicate cultures are shown. Error bars indicate standard deviation. Growth of *E. coli* was markedly inhibited by apo-transferrin.

Figure 8.

Effect of iron on the antimicrobial activity of transferrin. *E. coli* were grown in (A) MOPS medium with no added iron, or (B) MOPS medium with 1μM ferrous sulfate. Three treatments were used: 1μM apo-transferrin (blue), 1μM holo-transferrin (red), or an equivalent volume of buffer (gray). Bacterial growth is represented by the change in absorbance over the course of the experiment. Data are from three replicate cultures. Means $\pm$ SD are shown. Differences in means were assessed by performing a one-way ANOVA followed by a Dunnett test. Holo-transferrin had no detectable antibacterial activity, and iron

supplementation abolished the antibacterial effect of apo-transferrin. (* $P < 0.05$, *** $P < 0.001$)

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