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Histamine Release Factor from *Dermanyssus gallinae* (De Geer): Characterization and in vitro assessment as a protective antigen ☆

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ABSTRACT

A cDNA encoding a 174-amino-acid orthologue of a tick histamine release factor (HRF) was identified from the haematophagous poultry red mite *Dermanyssus gallinae*. The predicted *D. gallinae* HRF protein (*Dg*-HRF-1) sequence is highly conserved with the tick HRFs (identity 52–54%) and to a lesser degree with translationally controlled tumour proteins (TCTP) from mammals and other invertebrates (range 38–47%). Phylogenetically, *Dg*-HRF-1 partitions with the tick HRF clade suggesting a shared linage and potentially similar function(s). A recombinant *Dg*-HRF-1 protein (r*Dg*-HRF-1) was produced and shown to induce degranulation of rat peritoneal mast cells in vitro, confirming conservation of the histamine-releasing function in *D. gallinae*. Polyclonal antibodies were generated in rabbits and hens to r*Dg*-HRF-1. Western blotting demonstrated that native *Dg*-HRF is a soluble protein and immunohistochemical staining of mite sections revealed that the distribution of *Dg*-HRF, although ubiquitous, is more common in mite reproductive, digestive and synganglion tissues. A survey of hens housed continuously in a mite-infested commercial poultry unit failed to identify IgY specific for recombinant or native *Dg*-HRF, indicating that *Dg*-HRF is not exposed to the host during infestation/feeding and may therefore have potential as a vaccine using the concealed antigen approach. To test the protective capability of r*Dg*-HRF-1, fresh heparinised chicken blood was enriched with yolk-derived anti-*Dg*-HRF IgY antibodies and fed to semi-starved mites using an in vitro feeding system. A statistically significant increase in mortality was shown ($P = 0.004$) in mites fed with anti-*Dg*-HRF IgY after just one blood meal. The work presented here demonstrates, to our knowledge for the first time, the feasibility of vaccinating hens with recombinant *D. gallinae* antigens to control mite infestation and the potential of r*Dg*-HRF-1 as a vaccine antigen.

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1. Introduction

The blood-feeding poultry red mite *Dermanyssus gallinae* is the most economically important and widespread ectoparasite affecting commercial egg farming in Europe and causes considerable economic loss as a result of reduced production and mite control costs. The mite resides "off-host" in the cracks and crevices of the poultry house, becoming active and feeding at night. *Dermanyssus gallinae* is found in all types of egg production systems, but is present in greater numbers in free-range and barn systems than in battery units (Hoglund et al., 1995; Fiddes et al., 2005). Under ideal conditions *D. gallinae* can complete its life cycle (egg to egg) within 7 days and the mite population can double within 1 week (Maurer and Baumgartner, 1992). Mite numbers can reach in excess of 150,000 per hen, resulting in serious health and welfare issues for laying hens and potentially for poultry workers (Auger et al., 1979; Chauve, 1998; Kilpinen et al., 2005). In addition, *D. gallinae* has been implicated as a vector for several commercially important diseases such as Erysipelas and Salmonella (Chirico et al., 2003; Moro et al., 2005, 2007a,b). Chemical acaricide spraying is the current method generally employed for control; however the legislated withdrawal of toxic acaricides and the development of acaricide resistance (Beugnet et al., 1997; Nordenfors et al., 2001; Fiddes et al., 2005; Thind and Ford, 2007) has made alternative mite control strategies such as vaccination an attractive prospect.

Studies with Ixodid ticks have demonstrated the feasibility of using vaccination with "exposed" salivary antigens to reduce infestation (e.g. Iris protein; Prevot et al., 2007), however a protective natural immunity in hens based on exposed antigens of *D. gallinae* appears to be either absent or ineffective. "Concealed" antigens of

☆ *Note:* Nucleotide sequence data reported in this paper are available in the EMBL, GenBank and DDJB databases under the Accession No. FM179713.

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ectoparasites have shown most promise as exemplified by the commercially available TickGARD plus™/Gavac™ vaccines which are based on the concealed Bm86 midgut antigen from the cattle-tick *Rhipicephalus (Boophilus) microplus*. The TickGARD™ vaccine operates by generating antibodies to Bm86, which are ingested with a blood meal and bind to the antigen in tick gut epithelia inducing damage (Willadsen et al., 1989). An additional benefit of vaccination is the control of vector-borne diseases, such as Babesiosis and Anaplasmosis, by eliminating the tick vector (de la Fuente et al., 1998) and in some cases impairing the ability of ticks to become infected, thus reducing transmission (de la Fuente et al., 2006).

A histamine release factor (HRF), able to induce histamine release in rat basophils, was identified in several species of ticks (Mulenga et al., 2003a,b; Mulenga and Azad, 2005). The tick HRF protein is expressed in a variety of tissues including salivary gland, mid-gut, ovary and haemocytes and in all developmental stages, making it an ideal tick vaccine antigen candidate (Mulenga and Azad, 2005). Although the function of HRF in ticks is unclear, a related protein found in mammals, nematodes and other invertebrates (translationally-controlled tumour protein, TCTP; synonymous with HRF) has several described functions including: microtubule binding, cell growth and cell cycle progression, inhibition of apoptosis, histamine release and other cytokine-like activities; suggesting diverse regulatory functions (reviewed in Bommer and Thiele, 2004). The anti-malarial drug artemisinin is effective against *Plasmodium* and Schistosome parasites, and although the mechanism of the drug has yet to be elucidated, it has been shown that the parasites' HRF/TCTP proteins directly interact with artemisinin (Bhisutthibhan et al., 1998). A study by Walker et al. (2000) demonstrated that increased expression of *Plasmodium* HRF/TCTP is associated with a decreased susceptibility to artemisinin. This has lead to the proposal that HRF/TCTP is a valid target for drug or vaccine development for control of, or therapy for, these parasite species (Rao et al., 2002).

The aims of the work presented here were to characterize an orthologue of the tick HRF protein in *D. gallinae* and determine its potential as a vaccine antigen. We believe the work presented here is the first report demonstrating the potential of vaccination with recombinant *D. gallinae* antigens as a valid mite control strategy.

2. Materials and methods

2.1. Parasite material and eggs from infested hens

Dermanyssus gallinae mites and hen eggs were collected from a large mite-infested commercial battery unit which had been populated with the same Lohmann's Brown hens for 40 weeks. Large numbers (>5000) of mixed stage and gender mites were collected by scraping aggregated mites from the poultry cages and transferring into vented tissue culture flasks. The percentage of the mites held in the flask that had recently fed was immediately estimated by their appearance under light microscopy. Mites were only utilized if approximately 60% or more of the flask population had recently fed.

2.2. Cloning and sequencing of the D. gallinae HRF gene

Mites, free from poultry house detritus, were snap-frozen in liquid nitrogen and total RNA was immediately purified by homogenising mites in TRIzol® Reagent (Invitrogen) and enriched for mRNA using a poly(A)Purist™ Kit (Ambion) following the manufacturer's instructions. A cDNA expression library was constructed from 1 μg mRNA using the SMART™ cDNA Library Construction Kit (Clontech), employing the primer extension method. DNA sequencing of randomly selected clones resulted in the identification of a truncated cDNA encoding an orthologue of the tick HRF.

The complete coding sequence of the *D. gallinae* HRF gene (*Dg-hrf-1*) was obtained by rapid amplification of cDNA ends (RACE) using RACE-ready cDNAs generated with the SMART™ RACE cDNA Amplification Kit (Clontech) and the gene-specific oligonucleotide primers HRF-1F (5′-GAGGGCGAGGTTGTCTTGGCAGGC-3′) and HRF-1R (5′-GCCATGCACTGGTTCTTGAGTTCGG-3′) following the manufacturer's protocol. RACE PCR products were ligated into a pGEM T-Easy plasmid vector (Promega) and transformed into competent *Escherichia coli* cells (strain JM109, Promega). Individual positive clones were picked, based on blue/white selection, and grown overnight at 37 °C in Luria Bertani (LB) medium containing 100 $\mu g\ ml^{-1}$ of ampicillin. Plasmid DNA was purified using the QIAspin plasmid miniprep kit (Qiagen). The DNA sequence was obtained using automated sequencing with HRF-1F and HRF-1R primers (Moredun Functional Genomics Unit). The *Dg-hrf*-1 DNA sequence was deposited in EMBL under Accession No. FM179713.

2.3. Phylogenetic analysis

The predicted amino acid sequence of the full-length *Dg*-HRF-1 sequence was aligned with putative orthologues from other species of invertebrate and vertebrate organisms using the Clustal X algorithm (Jeanmougin et al., 1998) and a neighbour-joining tree drawn according to relationships inferred by the multiple sequence alignment. The neighbour joining tree was bootstrapped 1000 times using Clustal X (Jeanmougin et al., 1998) and the resulting tree viewed with TreeView (Page, 1996).

2.4. Expression of recombinant Dg-HRF-1 protein

The complete *Dg*-HRF-1 coding region was amplified by PCR with the oligonucleotide primers HRF-for (5′-AATTC*GGATCC*G**ATG**CTCATCTACAGGGACATC-3′) and HRF-rev (5′-CCGC*AAGCTT*GA CCTTCTCTTCCGCAAC-3′) that contain the ATG start codon (bold) and the restriction sites HindIII and BamHI (italicised) which allowed ligation into the cloning site of the pET22b(+) expression plasmid (Novagen) in the correct direction and in-frame with the 5′-*pel*B leader sequence and 3′-polyhistidine tag flanking the cloning site. PCR was performed using the Advantage® 2 PCR Kit (Clontech) with the cycling conditions: 25 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 90 s, followed by 1 cycle of 72 °C/5 min. The 544-bp PCR product was digested with HindIII and BamHI (Roche), purified using the QIAprep PCR cleanup kit (Qiagen) and inserted into the pET22b(+) vector using the Rapid Ligation kit (Promega).

Expression was carried out in BL21-CodonPlus®(DE3)-RIPL *E. coli* cells (Stratagene). Following induction with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), the recombinant *Dg*-HRF-1 (r*Dg*-HRF-1) protein was expressed at 30 °C for 3 h. Insoluble r*Dg*-HRF-1 protein was recovered from cell lysates prepared from inclusion body fractions. r*Dg*-HRF-1 was purified by binding to His-Trap™ HP columns (GE healthcare) using 8 M urea reducing conditions and eluted with increasing imidazole concentrations, according to the manufacturer's instructions. Solubilized r*Dg*-HRF-1 was refolded by dialysis against PBS with a decreasing gradient of urea.

2.5. Fractionation of D. gallinae proteins

Mites were successively homogenized in PBS, 0.1% Tween 20/PBS, 1% Triton X-100/PBS and 8 M urea in order to obtain protein fractions comprised of soluble, membrane-associated, integral-membrane and insoluble *D. gallinae* proteins, following a method described previously (Smith et al., 2002).

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