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Identification of protective linear B-cell epitopes on the subolesin/akirin orthologues of *Ornithodoros* spp. soft ticks

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ABSTRACT

Subolesin/akirin is a protective antigen that is highly conserved across hematophagous vector species and is therefore potentially useful for the development of a universal vaccine for vector control, including soft ticks. Recent results have shown that in *Ornithodoros erraticus* and *O. moubata* soft ticks, RNAi-mediated subolesin gene knockdown inhibits tick oviposition and fertility by more than 90%; however, vaccination with recombinant subolesins resulted in remarkably low protective efficacies (5–24.5% reduction in oviposition). Here we report that vaccination with subolesin recombinants induces non-protective antibodies mainly directed against immunodominant linear B-cell epitopes located on highly structured regions of the subolesin protein, probably unrelated to its biological activity, while leaving the unstructured/disordered regions unrecognized. Accordingly, for a new vaccine trial we designed four synthetic peptides (OE1, OE2, OM1 and OM2) from the unrecognized/disordered regions of the Ornithodoros subolesin sequences and coupled them to keyhole limpet haemocyanin (KLH). These KLH-peptide conjugates induced the synthesis of antibodies that recognized linear B-cell epitopes located on the unstructured loops of the subolesin protein and provided up to 70.1% and 83.1% vaccine efficacies in *O. erraticus* and *O. moubata*, respectively. These results show that the protective effect of subolesin-based vaccines is highly dependent on the particular epitope recognized by antibodies on the subolesin sequence and strongly suggest that the biological activity of subolesin is exerted through its unstructured regions. The results reported here contribute to our understanding of the mechanism of protection of subolesin-based vaccines and reveal novel protective peptides that could be included among the array of candidate antigens useful for developing anti-vector vaccines based on subolesin/akirin.

1. Introduction

The argasid ticks *Ornithodoros erraticus* and *Ornithodoros moubata* are important vectors of African Swine Fever (ASF) virus and several species of human relapsing fever borreliae in the Mediterranean, South and East Africa and Madagascar [1–7]. The presence of these argasid ticks in domestic and peridomestic environments contributes to the persistence of these diseases in endemic areas, and may facilitate the reintroduction and/or spreading of ASF into countries where it has been eradicated or where it has never existed previously [3,8–11]. Thus, the elimination of these ticks from synanthropic environments would greatly improve the prevention and control of such diseases.

Anti-tick vaccines have proved to be a feasible, cost-effective and environmental-friendly method for tick control, avoiding many of the drawbacks associated with the use of chemical acaricides for the control of tick infestations [12–14].

Some recently discovered tick protective antigens have been observed not only to reduce tick infestations but also to prevent the transmission of some tick-borne pathogens, paving the way for the development of vaccines with the dual target of controlling arthropod infestations and reducing the vectors' capacity to transmit pathogens affecting human and animal health [13,15].

One of such antigens is the so-called subolesin protein, initially discovered as a protective antigen in *Ixodes scapularis* [16] and subjected to intensive investigation since then [13,15,17–20]. Subolesin gene and protein sequences are highly conserved in ticks and are the orthologues of insect and vertebrate akirins, which are evolutionarily conserved proteins that function as transcription factors in the regulation of gene expression. The broad function of subolesin/akirin as transcription factors explains the profound effect of subolesin gene knockdown on tick physiology, affecting

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processes such as feeding, fertility and tissue development and resulting in the degeneration of tick guts, salivary glands, reproductive tissues and embryos. Moreover, subolesin has been found to be functionally important for tick innate immunity to pathogens and for the infection and multiplication in ticks of some pathogens such as *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Babesia bigemina* and *Borrelia burgdorferi* [17].

Vaccination with recombinant subolesin exerts an effective control of tick infestations by reducing tick numbers, weight and oviposition, as well as tick infection with different tick-borne pathogens. Additionally, subolesin/akirin share protective epitopes in different arthropod vectors, and vaccination with recombinant subolesin/akirin also exerts an effect on several vector species, including mosquitoes, sand flies, poultry red mites and sea lice, thus demonstrating the efficacy of subolesin/akirin-based vaccines for the control of vectors and vector-borne pathogens [15,17].

Although the exact mechanisms by which subolesin vaccines affect vector infestations, fertility and pathogen infection remain unknown, vaccination trials with subolesin/akirin have demonstrated that blood-sucking arthropods feeding on immunized hosts ingest sufficient amounts of specific antibodies to gain access to subolesin/akirin and disrupt its biological activity [17]. Additionally, some recent findings strongly suggest that the diverse effects of subolesin/akirin vaccines on tick infestation and tick infection by pathogens would depend on the particular protective epitopes recognized by the antibodies on the target protein [15,18,21].

Regarding the protective value of subolesin against soft ticks, we have recently observed that gene knockdown by RNAi of the subolesin orthologues in *O. erraticus* and *O. moubata* inhibited tick oviposition by up to 95%, but without affecting tick feeding and survival. By contrast, vaccination with the corresponding recombinant proteins induced strong humoral responses in rabbits, although these responses only reduced oviposition by between 5% and 25% [22].

At the time this low protective efficacy was attributed to the possibility that too few antibodies were reaching their intracellular target protein, but in light of the recent findings by de la Fuente et al. [17], Merino et al. [18] and Moreno-Cid et al. [15] it could be speculated that although sufficient amounts of antibodies could have reached their target, they could have been mainly directed to non-protective immunodominant epitopes, unrelated to the biological activity of the protein. If this were the case, then the induction of antibodies against the unrecognized parts of the protein might perhaps result in higher protection efficiencies.

The goal of the present work was to test this hypothesis by: (i) determining which epitopes were recognized by the antibodies from the vaccinated but little protected animals and (ii) assessing the protective efficacy of synthetic oligopeptides designed from regions of the subolesin amino acid sequence that were not recognized by the former antibodies and that are predicted to contain linear-B cell epitopes.

2. Materials and methods

2.1. Ticks and tick material

The *O. moubata* and *O. erraticus* ticks came from laboratory colonies maintained at the IRNASA, CSIC, Spain [1].

Tick saliva and salivary gland extracts (SGE) from adult unfed female ticks were obtained from both *Ornithodoros* species as described previously [1,23].

Gut extracts (GT) of both species were prepared from fasted females and from engorged females at 48 h post-feeding. The guts were dissected in phosphate buffered saline (PBS), pH 7.4, at 4 °C and rinsed several times in PBS [24]. Gut tissues were re-suspended in fresh PBS containing 5 μl/ml of complete EDTA-free proteinase inhibitor cocktail (Roche Diagnostics) and homogenized on ice using an Ultra-Turrax T10 disperser (IKA-Werke) and then sonicated 6 times for 30 s/each. Tissue homogenates were centrifuged for 20 min at 10,000 × *g* and 4 °C, and the 10,000 × *g* supernatants were recovered and centrifuged for 1 h at 100,000 × *g* and 4 °C. The new supernatants (soluble protein) were recovered and the pellets were re-suspended in PBS and also recovered (membrane protein).

Extracts of ovary soluble and membrane proteins were also prepared from fasted female ticks of each species using a similar protocol to that described for guts.

Protein concentration in all these extracts was measured using the BCA Protein Assay Reagent kit (Thermo-Fisher) and extracts were stored at −20 °C.

2.2. Polyclonal immune sera

The following sera were used for the pepscans described below.

Sera against recombinant subolesins from *O. erraticus* and *O. moubata* coming from previous work [22]. The sera from 2 groups of 3 rabbits immunized with each recombinant were collected at seven days after the last antigenic dose, titrated (>1/12,800), and stored at −80 °C. The pre-immune sera from these same animals were used as negative controls.

Anti-tick bite sera. These were obtained from 4 rabbits immunized by successive infestations with 100 specimens of each argasid species (2 rabbits/species), titrated against the SGE from the homologous species (>1/25.600), and stored at −80 °C.

Sera against subolesin synthetic peptides. The collection of these sera is described below in the vaccine trial section. Briefly, 4 peptides designed from the *Ornithodoros* subolesin sequences were coupled to keyhole limpet haemocyanin (KLH) (Sigma) and administered to rabbits. The anti-peptide sera were collected at seven days after the last antigen dose, titrated, and stored at −80 °C. The sera from 3 additional rabbits immunized with only KLH were used as negative controls.

2.3. Peptide arrays and epitope mapping (pepscan)

Subolesin peptide arrays were synthesized and spotted onto cellulose sheets at the Proteomics Service of the Centro Nacional de Biotecnología (Madrid, Spain). Each sheet contained 5 identical replicas of 51 and 49 overlapping 15-mer peptides with 12 amino acid overlaps, which covered the entire sequence of the *O. erraticus* (ADN66054.1) and *O. moubata* (ADN66053.1) subolesin orthologous proteins, respectively.

Pepscans were carried out following a protocol similar to that described by Rodriguez-Crespo et al. [25]. The sheets were submerged in ethanol to facilitate the hydration of the hydrophobic peptides, rinsed 3 times in 50 mM Tris, 150 mM NaCl (TBS) pH 7, and then blocked in TBS containing 5% skimmed milk, 5% sucrose and 0.05% Tween 20 (blocking solution) overnight at 4 °C. Then, they were washed in TBS containing 0.05% Tween 20 (washing buffer) and cut into strips. Each strip was incubated with the corresponding serum pool diluted 1/1.000 in blocking solution for 2 h at room temperature (RT). After 3 washes, the strips were incubated with peroxidase-conjugated anti-rabbit IgG diluted 1/10.000 in blocking solution for 2 h at RT. After six new washes, the strips were incubated with Immun-Star WesternC (BIO-RAD) for 5 min at RT.

Negative controls included strips processed without the primary antibodies or with pooled sera from non-immunized control animals or from animals immunized with KLH.

The strips were scanned in the ChemiDoc apparatus using Image Lab software (BIO-RAD). Image analysis was carried out using Image Master 2D platinum software (GE Healthcare Life sciences) including quantification of the spots volume. A peptide was