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Cutaneous hypersensitivity test to evaluate phage display anti-tick borne vaccine antigen candidates

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ABSTRACT

Early experiments performed by our group with the phage display technique revealed the potential for using epitope-displaying phages (mimotopes) as a tool for tick antigen discovery. Thus, as a preliminary study, inflammatory reactions induced by phage display tick-borne candidates were investigated by using the cutaneous hypersensitivity test. The profile of selected *Rhipicephalus microplus* mimotopes was assessed on tick field-exposed cattle and our data indicated a pattern similar to immediate hypersensitivity reaction and not a delayed immune response as expected. However, the wild-type phage inoculation surprisingly induced a strong immediate response on its own. Such reactions indicate that the wild-type phage may have hidden many of the potential reactions raised by the mimotopes. The study of the inflammatory reactions to these phage mimotopes in tick-infested hosts may provide basic information about the immune reaction. Finally, this work is of relevance for when considering research alternatives for finding and characterization of antigens by the phage display technique.

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1. Introduction

Rhipicephalus (*Boophilus*) *microplus*, the cattle tick, is one of the main constrains for animal production in several countries around the world (Jongejan and Uilenberg, 2004). Among several tick control methods, vaccination seems to be a promising alternative to acaricides (Canales et al., 2009; Willadsen, 2006), however antigen selection has proved to be a complex and expensive task. In this way, we have used strategies to discover novel proteins involved in the parasite–host relationship in large-scale analyses that have the power to survey the entire genome and identify targets with potential use in vaccine development (Cardoso et al., 2009; Cunha-Junior et al., 2010; Prudencio et al., 2010a,b). Early experiments performed by our group with the phage display technique permitted the identification, in a high-throughput manner, of *R. microplus* tick antigenic peptides fused on phages (mimotopes) and which were shown to be antigenic and to have the potential for eliciting a specific host immune response after immunization described by Prudencio (2008) and Prudencio et al. (2010a,b). Simultaneously, *in silico* analysis revealed protein features known to be important for antigen selection, which could lead to the identification of potential vaccine candidates (Prudencio et al., 2009). These studies demonstrated the epitope profile through screening of phage display peptide libraries and revealed the potential for using epitope-displaying phages as a tool for tick epitope discovery.

However, additional information on recognition and immune reaction to these clones in the target host is necessary for the proper evaluation of new anti-tick vaccine candidate antigens. A fast method to provide a broad overview of bovine immune response to candidate antigens is the evaluation of cutaneous hypersensitivity at intradermal inoculation sites of antigens on animals known to be either resistant or susceptible to tick infestations.

Hypersensitivity reactions are commonly classified according to the type of immune response and the effector mechanism responsible for cell and tissue injury (Abbas et al., 2000); hence cutaneous hypersensitivity reactions elicited by intradermal testing can be used to broadly determine prevailing immune responses to antigens in hosts (Ferreira et al., 2003). Inflammatory responses of hosts against ticks' extracts are documented for dogs (Mukai et al., 2002a,b; Szabo et al., 1995), bovines (Bechara et al., 2000), and horses (Szabo et al., 2004). At the same time, the antigens or more specifically the epitopes that induce these natural reactions are not characterized. This likely includes those phage-displayed peptides that mimic tick proteins exposed to the host via the salivary glands as a result of successful tick feeding.

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In the present work, in an attempt to understand the immune response to some of these phage-borne peptides, cutaneous hypersensitivity reactions to intradermal phage-displayed tick mimotopes of *R. microplus* tick-exposed cattle were assessed. To our best knowledge, intradermal testing has not been previously used to evaluate immune responses to phage-displayed peptides. Moreover, if an alternative control measure such as vaccination against *R. microplus* is to be developed from these mimotopes, information on target host reaction to them is fundamental.

2. Material and methods

2.1. Animals and treatment

Six Holstein Friesian (*Bos taurus*) and Nelore (*Bos indicus*) cross-breed and eight pure breed Nelore young animals aged 18 months were used in this trial. Both groups came from *R. microplus* tick -infested areas of the Uberlândia municipality, Minas Gerais State, Brazil. Furthermore, several months prior to the skin testing experiment all animals were maintained in the same *R. microplus* tick -infested pasture and throughout the skin testing in the same paddock under the same conditions.

2.2. Tick counting

Standard tick counts of all young animals were undertaken weekly, for 10 weeks, prior to the skin testing as described (Utech and Wharton, 1982). Briefly, every tick between 4.5 and 8 mm (semi-engorged female ticks) on one side of the animal was counted.

2.3. Preparation of phage display tick-borne peptides

The phage display tick-borne peptides were previously selected against chicken polyclonal IgG antibodies raised against proteins of *R. microplus*, as described by Prudencio (2008) and Prudencio et al. (2010a,b). After the biopanning, the Mimotope C1 (SVQERYYAFWST) was selected against chicken polyclonal IgG antibodies raised against adult proteins of *R. microplus* and amplified as described previously. Individual Mimotopes C2 (DAWKMRLSQMYD), and C3 (IDPLMFKYWYNM) were selected from antibodies anti-larval proteins of *R. microplus*. After biopannings, the phage clones were amplified as described previously (Prudencio et al. 2010a,b). The clone C4 (CPLEKSHLC), also selected from antibodies anti-larval proteins of *R. microplus,* was described (Prudencio, 2008). Briefly, the phage-displayed peptides C1, C2 and C3 contain duodecapeptides and the clone C4 contains heptapeptides plus two flanking cysteines followed by a short spacer of Gly–Gly–Gly–Ser sequence fused to the N-terminus of the M13 phage minor coat protein III. All five copies of pIII contain the amino-terminal peptides. The selected phage clones were amplified by infecting an early-log-phase culture of *Escherichia coli* ER2738 and incubated with shaking at 37 °C for 8 h. Culture was centrifugated and the supernatant was precipitated twice by adding 0.2 volume of polyethylene glycol solution (20% PEG-8000, 2.5 M NaCl), incubated at 4 °C overnight before being centrifugated at 15,000×*g* for 10 min at 4 °C. The precipitated phage was resuspended in 2 mL of PBS. The supernatant was collected and dialyzed in PBS at 4 °C. The phage titer was estimated in plaque forming units (pfu) for the M13 virus by conventional titer (Barbas et al., 2001). The final phage stock was stored at 4 °C.

2.4. Intradermal test

Intradermal testing was performed on physically restrained animals after the last tick count. The precipitated phages were diluted in 0.2 mL of PBS. Phage-displayed recombinant mimotopes clones C1, C2, C3, and C4 were injected into the dermis of each calf at separate sites of the previously shaved neck of young animals (10^{13}, 10^{12}, and 10^{11} pfu in 0.2 mL PBS). Wild-type phage (10^{13}, 10^{12}, and 10^{11} pfu) was used as negative control, and larval tick extract as positive control (10 μg). Considering prior experience with dogs (Szabo et al., 1995), bovines (Bechara et al., 2000), and horses (Szabo et al., 2004), the same phage-displayed peptides were also injected into the dermis of either the right or left ear of each animal (10^{12} pfu in 0.1 mL PBS). Reactions were evaluated by measuring skin thickness at 1, 2, 5, 7, 24, 48, 72, and 96 h post-injection with a caliper.

2.5. Statistics

The global tick counting of *Bos taurus* and *Bos indicus* were compared by analysis of variance. One-way ANOVA with unpaired *t* test with Welch correction was performed using GraphPad InStat version 3.0a for Macintosh, GraphPad Software, San Diego California USA (www.graphpad.com). Intradermal tests results were expressed as mean percentage of increase in skin thickness in relation to pre-inoculation values. PBS-induced skin thickness increase was considered a non-specific alteration of the injection, and was therefore subtracted from all other values of the same animal.

3. Results and discussion

Only young animals displaying the lowest and highest infestations (as determined by analysis of variance and means of tick counts) within each breed were selected for skin tests (Fig. 1). The four selected young animals (extreme counts of each category of the 12 animals studied) were thus categorized as tick-resistant (HR) or susceptible (HS) Holstein cross-breed (*Bos taurus* and *Bos indicus*), or tick-resistant (NR) or susceptible (NS) Nelore (*Bos indicus*). This selection aimed at distinguishing immune recognition of mimotopes by susceptible and resistant animals from each breed. It was supposed that those mimotopes to which resistant young animals reacted more pronouncedly and the type of reaction to it (either immediate or delayed) would indicate antigen and immune reaction mechanism in need of reinforcement in susceptible animals. Such associations between tick resistant and susceptible Holstein cross or Nelore were considered a preliminary and qualitative trial in the evaluation of reactions induced by phage display tick-borne mimotopes as there is, up to our knowledge, no similar work in the literature.

Overall, it was observed that Holstein cross-breed animals had consistently higher tick counts with two clear peaks (count 5 and counts 8–9). The mean number of ticks counted on the pure *Bos indicus* animals was 11.5 ticks per side, while the mean number of ticks observed on the cross-breed animals was 39.6 per side. It should be noted, however, that HR and NS animals had largely similar tick counts.

Next skin tests were performed to observe if the animals displayed a corresponding variation in cutaneous reactions. All animals developed an immediate reaction (within 2 h of the injection) to unfed larval extract in the neck (Fig. 2) and ear (data not shown). This reaction was more pronounced in the neck of cross-breed animals with an increase of approximately 120% in skin thickness, while both Nelore animals had an increase of approximately 60%. In the ear inoculation site (data not shown), the immediate reaction was less noticeable, with the HS calf presenting the highest reaction (37% of thickness increase), HR and NS displaying an intermediate reaction with 26% of increase, and NR showing a small 9% increase.