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Vaccination of lambs against *Haemonchus contortus* infection with a somatic protein (Hc23) from adult helminths

Elshaima M. Fawzi, María Elena González-Sánchez, María Jesús Corral, Montserrat Cuquerella, José M. Alunda *

Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

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ABSTRACT

A somatic protein from adult *Haemonchus contortus* (Hc23), the most abundant component in a low molecular weight fraction with known immunizing effect against experimental haemonchosis, has been purified by immunochromatography. The immunoprophylactic value of Hc23 was tested in groups of 5–6 months old Assaf lambs using $Al(OH)_3$ or *Escherichia coli* lipopolysaccharide + inactivated *Propionibacterium acnes* as adjuvant and the results compared with uninfected control, uninfected and challenged or infected and challenged lambs. Immunization with Hc23 in either adjuvant elicited significant reductions in fecal egg counts after challenge with 15,000 L3s (70.67%–85.64%, respectively) and reduced (67.1% and 86%) abomasal worm counts (45 days post-challenge). Immunized lambs displayed higher peripheral eosinophil counts, were less anaemic and had weight gains than challenged controls. The results suggest that the Hc23 antigen can induce a partially protective response against haemonchosis in lambs.

1. Introduction

The abomasal nematode *Haemonchus contortus* is a highly pathogenic blood feeding parasite of small ruminants, especially lambs. The L4 and adult stages of the parasite attach to the abomasal mucosa of the host, resulting in anemia which can be severe enough to cause death. *Haemonchus contortus* infections have a wide range of clinical courses from acute, often fatal, especially for young lambs, to chronic forms, particularly in older lambs and sheep. Mortality ranging from 30–50% has been reported in lambs and kids in acute cases (Aumont et al., 1997; Baker, 1997). Production losses in the chronic subclinical infection include weight loss, reduced weight gain, reproductive inefficiency (decreased fertility), reduction of wool growth and quality, and decline in the milk yield. Globally, haemonchosis is by far the most important nematode infection of small ruminants (Waller and Chandrawathani, 2005) and represents ~15% of all gastrointestinal diseases of these species worldwide (http://www.fao.org). Control of the infection relies mainly on the use of anthelmintic drugs, although high levels of anthelmintic resistance (AR) have been described in the major parasites affecting ruminants (Echevarria et al., 1996; Waller, 1997; Kaplan, 2004). The appearance of AR has become a common phenomenon, including multiple drug resistance against the three major classes of anthelmintic drugs (benzimidazoles, imidothiazoles and macrocyclic lactones) that compromise successful chemotherapy (Jackson and Coop, 2000; Kaminsky, 2003; Coles et al., 2006; Wrigley et al., 2006).

Among possible alternative strategies to control *H. contortus*, vaccine development is the most investigated method (Miller and Horohov, 2006). To date no commercial vaccines are available for any gastro-intestinal nematode (GIN). Several types of proteins have been used to immunize lambs or kid goats against GIN, using both hidden and exposed antigens (Ags). Hidden Ags are mainly enzymes from the gut of the parasite and are not recognized during infection. Vaccination induces high levels of antibodies (Abs) which probably neutralize these enzymes. Natural Ags are recognized during the infection and include excretory/secretory (ES), surface and somatic Ags and can be effective against both blood and non-blood feeding nematodes (Newton and Meeusen, 2003). An advantage of the so-called natural antigens is the possibility of natural boosting whilst being continuously infected on pasture.

Variable results have been obtained in vaccination trials with *Haemonchus contortus* but some Ags have achieved notable protection levels. Thus Schallig and van Leeuwen (1997) found protection between 75% and 85% with an enriched fraction of ES Ags containing two proteins (15 and 24 KDa). Newton and Munn (1999)

* Corresponding author. Tel.: +34 913943701; fax: +34 913943908.
E-mail address: jmalunda@ucm.es (J.M. Alunda).

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achieved over 90% protection with H11 from L4 and adult worms and Smith et al. (2003) observed reductions in egg excretion and worm counts of 93% and 72% with H-gal-GP. Comparable values have been obtained more recently (Cachat et al., 2010). Lower values of protection (50%) have been obtained with cystine proteases (Bakker et al., 2004).

Previous experiments showed that the low molecular weight fraction p26/23, obtained from soluble extracts of adult *H. contortus*, conferred notable protection against experimental haemonchosis in lambs provided that Freund's adjuvant was employed (Domínguez-Toraño et al., 2000). However, this adjuvant is not acceptable and the protective components of this fraction were not identified with certainty. Our aim was to purify native somatic protein Hc23 from the p26/23 fraction and to determine its protective ability when formulated with aluminum hydroxide ($Al(OH)_3$) or a bacterial immune modulator preparation (lipopolysaccharide of *Escherichia coli* (LPS) + inactivated *Propionibacterium acnes*) as adjuvant.

2. Materials and methods

2.1. Parasites

The *H. contortus* strain used, originally supplied by Merck, Sharp and Dohme (Madrid, Spain), has been maintained for the last 20 years in the Department of Animal Health, Universidad Complutense de Madrid (UCM), Spain by serial passage in lambs. L3s were obtained by fecal culture at 26 °C, after baermanization of the feces and partial purification on filter paper (MAFF, 1971).

2.2. Purification of native protein Hc23

Adult *H. contortus* were obtained from the abomasums of lambs infected with a pure isolate of the parasite. Male and female worms in PBS were subjected to eight cycles of freezing/thawing (−20 °C/room temperature), homogenized in a glass-in-glass homogenizer and centrifuged (30,000*g*, 4 °C, 30 min; Klesius et al., 1984); the supernatant was recovered and stored at −80 °C (adult soluble extract, ASE) and the protein concentration was determined with the Bradford method (Bradford, 1976). Purification of native Hc23 protein from *H. contortus* was carried out by immunoaffinity chromatography using polyclonal Ab raised against a non-protective recombinant form of p26/23 (rHcp26/23) in rabbits (García-Coiradas et al., 2010) following Fitzgerald et al. (2011). A 3 mL column of protein A agarose was prepared to purify 10–20 mg of Ab. The column was pre-equilibrated with five volumes of 100 mM Tris–HCl pH 8 buffer and the sample was clarified by centrifugation (10 min at 10,000*g*, 4 °C). The sample was loaded into the column, washed with 100 mM Tris–HCl pH 8 buffer and the bound immunoglobulins (Igs) eluted with 100 mM Glycine pH 3 and neutralized with 1 M Tris–HCl pH 8. The Ig-containing fractions were identified (by absorbance at 280 nm (A280)) and the purified Igs was dialyzed against PBS. The polyclonal Abs were obtained and the concentration was determined (Bradford, 1976). Purified Abs were mixed with buffer (0.2 M ammonium bicarbonate and 0.5 M NaCl pH 8.3) and were coupled to a N-hydroxysuccinimide (NHS) activated Sepharose HP column (GE Healthcare, USA) overnight at 4 °C. Uncoupled Abs were removed with blocking buffer (0.2 M Tris–HCl and 0.5 M NaCl pH 8.3) and the column was washed (0.1 M sodium acetate and 0.5 M NaCl pH 4). *Haemonchus contortus* ASE was loaded into the column and left overnight at 4 °C. The flow-through was collected and the column was washed with equilibration buffer (PBS) until A280 returned to the baseline. Protein was eluted by 0.1 M glycine-HCl pH 2.25 and neutralized with 2 M Tris–HCl, pH 8.6 (Subramanian, 2002). Representative fractions (flow-through, washing with PBS and fractions containing Hc23) were analyzed by PAGE under denaturing and reducing conditions (SDS–PAGE 12.5%) using 0.025 M Tris, 0.192 M glycine and 0.1% SDS as electrophoresis buffer. For two dimensional (2D) electrophoresis, lyophilized samples were resuspended in MilliQ water (200 µL), precipitated with 2D-Clean Up (GE Healthcare), centrifuged in acetone and resuspended in strip buffer (see below) up to a final concentration of 50 µg/sample. The 2D electrophoresis was performed using BioRad equipment. For the first dimension, 7 cm immobilized pH gradient (IPG) strips pH 3–11 NL (GE Healthcare) were used. They were hydrated, containing sample, with 7 M urea, 2 M thiourea, 4% (w/v) 3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate, CHAPS), 100 mM DeStreak, and 2% pharmalytes at pH 3–11, overnight. Isoelectric focusing (IEF) was performed at 20 °C using the following program: 120 V for 15 min, 500 V for 15 min, 500–1000 V in gradient for 2 h, 1000–500 V in gradient for 2 h, and 5000 V for 2 h. Subsequently, strips were equilibrated for 12 min in reducing solution (6 M urea, 50 mM Tris–HCl at pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS, and 2% (w/v) DTT) and then for 5 min in alkylating solution (6 M urea, 50 mM Tris-HCl at pH 6.8, 30% (v/v) glycerol, 2% (w/v) SDS and 2.5% (w/v) iodoacetamide). The second-dimension SDS–PAGE was run on homogeneous 12.5% T and 2.6% C polyacrylamide gels.

Electrophoresis was carried out at room temperature, 100 V/gel for 2 h. To visualize proteins, 2D: gels were stained following the colloidal Coomassie blue protocol. For western blot assays, 2D gels were transferred to a polyvinylidene difluoride (PVDF) membrane. After washing and blocking, the membrane was incubated with pooled sera from group III (GIII) lambs which had been diluted 1/100 for 3 h at 37 °C. The conjugate was horseradish peroxidase (HRP)-labeled donkey anti-sheep IgG (Sigma–Aldrich, USA) diluted 1/1000 (1 h at 37 °C). Color was developed with 4-chloro-1-naphtol (0.5 mg/mL). Molecular weight (MW) markers were from GE Healthcare and Bio-Rad (USA). 2D electrophoresis, MS and Peptide Mass Fingerprinting were carried out by the Proteomics Services of the UCM. Samples were digested (with trypsin and *Staphylococcus aureus* Endo V8) and homologies of mass maps were checked against Protein Prospector (http://prospector.ucs.edu) and Source Database: NCBI Resources, NIH, Bethesda MD, USA, Matrix Science, MASCOT (http://www.matrixscience.com).

2.3. Lambs and experimental design

The experimental design and procedures were approved by the Ethical Committee of the UCM.

Female 4–5 months old Assaf lambs were obtained from a local producer (Finca La Mora, Pozuelo del Rey, Madrid, Spain). Coproscopical analyses, carried out immediately after their arrival, showed a slight coccidial infection and all animals were treated with Borgal® 24% Sulfadoxine-Trimetoprim (Virbac, Spain) (5 mL/animal i.m., two doses at 48 h intervals). Lambs were maintained under *H. contortus*-free conditions at the Faculty of Veterinary Medicine, UCM where they were fed commercial pellets (Rubio Sanidad y Alimentación Animal, Madrid, Spain), hay and tap water ad libitum. The lambs (34.8 ± 5.9 kg) were allocated to four groups of seven and one of six, and balanced for weight. Group I (GI) was immunized with Hc23 (three doses of 100 µg of Hc23 + 0.9 mL of $Al(OH)_3$ gel colloidal suspension (13 mg/mL) (Sigma-Aldrich) on days −42, −28 and −14. GII was vaccinated with 100 µg of Hc23 and a bacterial adjuvant (LPS of *E.coli* + *Propionibacterium acnes*) (Lab. Calier, Spain) (1 mL/10 kg of live weight (lw)) per dose, on days −2, 0, 7 and 14 of the experiment) following the manufacturer's recommendations. Immunizing doses were administered by i.m. and s.c. injections in the legs and the groin, respectively. GIII was unvaccinated and uninfected, GIV was unvaccinated but

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