Effects of immunization of Pelibuey lambs with *Oestrus ovis* digestive tract protein extracts on larval establishment and development

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Abstract

Larval midgut proteins of hematophagous parasites contain strong antigens that can be used for host immunization. This concept has been applied for immunization of Pelibuey sheep against *Oestrus ovis* L. (Diptera: Oestridae). The aim of this study was to examine the effect of immunization on larval establishment (LE) and development. Immunized lambs (*I*, *n* = 6) received two injections of crude gut membrane protein extracts (GMPE) from third instar larvae with Freund’s incomplete adjuvant (FIA) on days 0 (Day of first immunization) and 21 (0.4 and 0.45 mg GMPE/lamb, respectively). The control group (*C*, *n* = 5) received physiological saline with FIA. Lambs were challenged with first instars on Day 29 (20 larvae) and Day 43 (25 larvae). Blood samples were collected biweekly and IgG titers were analyzed by ELISA. All lambs were slaughtered on Day 90 and number of larvae recovered, larval stage and larval weight were recorded at necropsy. No significant effect of immunization on LE (*C* = 28.9%; *I* = 31.0% *P* > 0.05) was observed. Antibody titers were higher in the immunized group on Day 28 (*P* < 0.05), but subsequently similar in both groups. Larval physiological age and weight were also significantly (*P* < 0.05) affected by immunization. Immunization of Pelibuey lambs with GMPE did not affect LE but did delay *O. ovis* larval development.

Keywords: *Oestrus ovis*; Myiasis; Midgut antigen; Immunization; Antibody; Sheep

1. Introduction

*Oestrus ovis* L. (Diptera: Oestridae) larvae parasitize the nasal cavities of sheep and goats causing extensive production losses and affecting the well-being of both hosts around the world. Vaccinating with antigens from the arthropod parasite saliva or digestive system can protect hosts or interfere with the parasite viability (Titus et al., 2006). *O. ovis* larvae excrete and secrete products (mainly enzymes) that are in contact with the host mucosa and are used to digest food in their digestive system. These products have been used for diagnosis and immunization trials in sheep (Tabouret et al., 2001). Antigens found in the larval midgut peritrophic membrane are called “hidden or concealed antigens” because they are not in contact with the host during natural infections (Smith, 1993). In parasitic
nematodes, most of these proteins have been identified as proteases found in the microvilli surface of intestinal cells and probably are needed for nutrient digestion (Douglas et al., 1996; Knox et al., 2003). The mechanism of action appears to involve recognizing and blocking larval digestive proteins by host antibodies ingested by the parasite. As a result, decreasing larval developmental rates, egg production, and eventually death occurred (Newton and Meeusen, 2003). In a field trial (Smith et al., 2001) using gut membrane antigens from Haemonchus contortus Rudolphi (Strongylida: Trychostrongylidae), parasite egg output was reduced by 82%, and host anaemia and deaths due to haemonchosis were reduced as well. Knox et al. (2005) reported 70–95% reductions in fecal egg output of H. contortus in immunized sheep. Frugère et al. (2000) observed lower weights of O. ovis larvae recovered from lambs immunized with larval excretory–secretory products (ESP). Reduced weight of O. ovis mature third instars (<280 mg) may compromise subsequent survival of the free stages (Cepeda-Palacios et al., 2000). To date, O. ovis antigens from the larval intestine have not been tested in immunization trials. The aim of this study was to evaluate the effects of immunization of Pelibuey sheep with gut membrane proteins extracts (GMPE) of O. ovis on larval establishment and development.

2. Materials and methods

2.1. Larval collection and antigen preparation

O. ovis larvae were collected from the sinusal cavities of slaughtered sheep originating from several farms located in La Paz, B.C.S., Mexico. Gut antigens were extracted from third instars because heavier larvae have more developed digestive tracts facilitating the midgut dissection. Furthermore, L3 larvae yielded more protein tissue for analytical and immunization procedures. After collection, larvae were individually washed in sterile water and frozen at −20°C until dissection. A total of 22 third instar larvae were dissected under a stereoscope at 4°C using phosphate buffer (PBS) pH 7.2 as dissection medium. For this purpose, larvae were fixed dorsally with entomological pins in Petri dishes, and the ventral coelomic wall was removed to expose the internal organs. The salivary glands, fat body, and tracheal tissue surrounding the digestive system were removed, then the intestine was cut at the foregut–midgut junction at the point of insertion of the Malphighian tubules (to eliminate the hindgut segment). Midgut tissue was stored at −70°C in 1 mL conical tubes containing sterile PBS pH 7.2.

Gut membrane proteins were isolated using the method described by Smith (1993). Briefly, larval digestive tissue was thawed and macerated in PBS pH 7.2 medium and centrifuged at 10,000 × g at 4°C for 10 min. The supernatant was discarded and the pellet washed twice with PBS + Tween 20 (BioRad, Hercules, CA) 0.1% (1.5 mL) and vortexed, discarding the supernatants. The pellet was resuspended in PBS + Triton X-100 (BioRad) 2% and maintained under slow shaking for 2 h at 4°C; then centrifuged. The supernatant was recovered, filtered using a 0.2 μm pore membrane filter (VWR, West Chester, PA) and stored at −70°C. Protein concentration of the filtrate was determined by the Bradford technique with bovine serum albumin used as a standard (Bradford, 1976). Proteolytic activity of the antigenic suspension with GMPE was positively tested using the degradation of Azo Dye Impregnated Collagen technique (Azocoll, SIGMA, St. Louis, MO) as described by Tamashiro et al. (1987).

2.2. Electrophoresis analysis

Proteins from GMPE extracts were resolved on discontinuous gels (4–12%) SDS-PAGE following the method of Laemmli (1970) under reducing conditions (2-β-mercaptoethanol, 95°C for 5 min). Electrophoresis was performed at 80/100 V for 30 min/2 h. Gels were stained with Coomassie blue R-250 and distilled with a solution containing methanol (40%) and acetic acid (10%). A low molecular weight marker (ranging 14.4–97.4 kDa, BioRad) was used to estimate the molecular weight of resolved proteins.

2.3. Experimental procedure

Eleven 1-year-old haired Pelibuey male lambs (average body weight 37 kg) were reared in pen allotments in La Paz, B.C.S., Mexico. During the trial, lambs were fed alfalfa hay, concentrate, mineral blocks, and water ad libitum. After weaning, lambs were kept isolated from flying insects to avoid natural field infections with O. ovis. Lambs were randomly assigned to control (n = 5) and immunized (n = 6) groups. Immunized lambs received two intramuscular (IM) injections in the neck, 3 weeks apart (Day 0, Day 21) in Freund’s incomplete adjuvant (FIA). Total amount of GMPE protein injected to each lamb was 0.85 mg (0.4 mg in the first injection and 0.45 mg in the second one). FIA was used because it elicits both strong humoral and cellular responses (Audibert and Lise, 1993; East et al., 1992). Control group lambs received

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two IM injections of physiological saline solution with FIA. All lambs were slaughtered on Day 90 for post-mortem examination, according to local sanitary and humanitarian regulations.

2.4. Artificial infections

Gravid *O. ovis* flies were manually caught in the field, in the vicinity of an extensively grazed goat herd (300 km north, La Paz, Mexico) that was maintained in a small pen during the warmest time of the day. Infective female *O. ovis* flies were noticeable by being very active at air temperatures of 20–35 °C searching for suitable hosts (Cepeda-Palacios and Scholl, 2000). First instars were obtained by softly squeezing the abdomen of gravid flies. Between 80 and 250 larvae per fly were obtained. Prior to experimental infections, larval viability (vigorously moving larvae) was observed with backlighting in physiological saline, using a stereo-scope. For dose preparation, live first instars were mixed, counted, and pooled into appropriate doses for inoculation. Experimental challenge was carried out on Day 29 (20 larvae per lamb) and Day 43 (25 larvae per lamb), i.e. 8 and 22 days after the second immunization. Larvae were deposited deep in the left nostril of each lamb with Pasteur pipettes. Their establishment was confirmed by direct observation of their movements on the nasal mucosa. After the first infection, all lambs were examined daily to detect clinical signs of oestrosis.

2.5. Antibody kinetics

The kinetics of IgG antibodies against *O. ovis* GMPE was monitored biweekly by ELISA from Day 0 to Day 90. Crude extracts of L2 larvae were diluted in carbonate buffer (pH 9.6) at 2 μg mL⁻¹, distributed in 96 well plates (Nunclon, polylabo) and incubated for 1 h at 37 °C then overnight at 4 °C. The wells were washed three times with PBST (0.01 M phosphate, 0.15 M sodium chloride, pH 7.2, and 0.1% Tween 20). The antigen coated wells were then incubated for 30 min with a 10% skimmed milk solution at 37 °C. Triplicate serum samples diluted in PBST (1:200) were incubated for 60 min at 37 °C. The plates were washed three times with PBST before the addition of horse-radish peroxidase-conjugated donkey anti-sheep IgG (Sigma A3415) diluted (1:2000) in PBST (60 min of incubation, 37 °C). Three final washes with PBST were carried out before addition and incubation at 37 °C of 100 μL per well of the chromogen (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid))diammonium). The reaction was stopped after 1 h and the optical densities (OD) determined with a spectrophotometer by measuring the absorbance at 450 nm (Frugère et al., 2000). Negative (lambs never exposed to *O. ovis* infection) and positive sera were used as controls. An antibody percentage was calculated for each serum sample by comparison with a positive reference serum (artificially infected sheep) as follows:

\[
\text{Antibodies} \, (\%) = \frac{\text{OD (serum sample)} - \text{OD (negative control)}}{\text{OD (positive control)} - \text{OD (negative control)}} \times 100.
\]

2.6. Control of immunization efficiency

Necropsy of lambs was performed by cutting the heads through the medial line. Larvae were collected from the nasal septum, the turbinates, the ethmoid bone, and the sinusal cavities.

2.7. Larval development

*O. ovis* larval development was estimated by either larval physiological age score or larval live weight. A 12-point physiological age classification for *O. ovis* larvae (Cepeda-Palacios et al., 1999), including early L1 (score 1) to fully mature L3 larvae (score 12) was used. Likewise, live larval weight was recorded individually using an analytical balance (minimum division 0.01 mg).

It is noteworthy that a greater percentage of first instar larvae collected at necropsy from a particular host usually increased its respective larval burden size, but not its average larval burden age or weight. Conversely, L2 and L3 larvae were physiologically older and heavier than L1 larvae, but they were usually hosted in lower percentages (see Yilma and Dorchies, 1993). In order to get a better estimation of larval development, live larval weight, larval stage development, and number of larvae data were included in a larval development index (LDI). A LDI per group-treatment was calculated as follows:

\[
\text{LDI} = \frac{\sum_{i=1}^{n} W_i S_i}{n}
\]

where *W* is live weight of the *i* larva collected from the lambs in each group at necropsy, *S* the larval stage (1, 2, or 3) of the *i* larva, and *n* is total number of larvae collected from each group. Thus, LDI was increased by larval weight and stage, and decreased by the number of larvae.
2.8. Statistical analysis

Antibody levels, larval weight, larval burden (number of larvae collected at necropsy per lamb), and LDI were compared between groups by Student’s t-test for groups with unequal repetition number (Steel and Torrie, 1981). Establishment rate (number of larvae collected at necropsy/total number of larvae inoculated) were compared using a Chi-square analysis. All statistical procedures were performed using the Statistica software (Stat-soft., 1998).

3. Results

3.1. Proteolytic activity and electrophoresis pattern of antigens

Enzymatic activity of GMPE measured by light spectroscopy (absorbance 0.428 ± 0.060) indicated high proteolytic activity on collagen. This test demonstrated that antigen proteins from intestinal extracts were not degraded at the time of immunization. Molecular weights of proteins resolved on polyacrylamide gels electrophoresis under denaturing conditions are shown in Fig. 1. Five major bands, ranging 58–28 kDa (58, 55, 52, 38, and 28) were observed, weak bands from 150 to 22 kDa were also noted.

3.2. Clinical signs of oestrosis

All experimental lambs showed symptoms of discomfort (sneezing, frequent movement of the head, and restlessness) after the first larval inoculation and during the following 4 days. No severe symptoms of oestrosis were observed in control or immunized lambs during the rest of the experimental period. However, nasal and sinusal mucosae were observed to be mono or bilaterally inflamed at necropsy in both groups, in some cases with local secretion of mucus and pus.

3.3. Antibody response

Specific IgG production as the percentage of positive controls are shown in Fig. 2A and B. Antibody response patterns were similar in both groups after the first experimental infection and remained similar through the end of the experiment. IgG titers increased dramatically in both groups only after the first and second experimental infections and reached maximum levels on Day 90, at the end of the experiment.

3.4. Larval establishment and development

On average, control, and immunized lambs harbored similar burdens of larvae of all stages (Table 1). In general, control, and immunized lambs hosted, respectively, 3.2 and 1.5 L3 larvae at the end of the experiment. Average larval weight was different for the control and immunized groups ($P < 0.01$). When weight of the growing L2–L3 stages were pooled and compared, a significant difference ($P < 0.01$) was found between controls (0.206 ± 0.190 g) and immunized (0.078 ± 0.160 g, data not shown in tables). Larval establishment rate recorded at necropsy was similar in control (28.9%) and immunized (31.0%) groups ($P > 0.05$). The proportion of third instar larvae in controls 24.6% (16/65) was noticeably higher than in immunized lambs 10.6% (9/84).
In group-pooled data, the ILD in the control group (0.249/0.47) was about 5.2 times greater (P < 0.01) than in the immunized group (0.048/0.22). This means that even though larval burden was similar in both groups of lambs, developmental rate was slower in larvae hosted by immunized lambs. Average physiological larval age in controls was 4.5 ± 2.7 (which corresponds to the late second instar period, according to Cepeda-Palacios et al., 1999) and 3.7 ± 3.1 (corresponding to early second instar period) in immunized lambs.

### Table 1

<table>
<thead>
<tr>
<th>Groupa</th>
<th>Larval stage</th>
<th>n</th>
<th>Mean number</th>
<th>S.D.</th>
<th>Mean weight (g)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control n = 5 lambs</td>
<td>L1</td>
<td>39</td>
<td>7.8</td>
<td>4.8</td>
<td>0.00046</td>
<td>0.0003</td>
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<tr>
<td></td>
<td>L2</td>
<td>10</td>
<td>2.0</td>
<td>1.4</td>
<td>0.01390</td>
<td>0.0138</td>
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<tr>
<td></td>
<td>L3</td>
<td>16</td>
<td>3.2</td>
<td>3.8</td>
<td>0.32600</td>
<td>0.1429</td>
</tr>
<tr>
<td>Burden mean</td>
<td></td>
<td>65</td>
<td>13.0a</td>
<td>2.0</td>
<td>0.08402a</td>
<td>0.1575</td>
</tr>
<tr>
<td>Immunized n = 6 lambs</td>
<td>L1</td>
<td>62</td>
<td>10.3</td>
<td>6.1</td>
<td>0.00042</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>L2</td>
<td>13</td>
<td>2.2</td>
<td>1.7</td>
<td>0.00640</td>
<td>0.0044</td>
</tr>
<tr>
<td></td>
<td>L3</td>
<td>9</td>
<td>1.5</td>
<td>1.4</td>
<td>0.31800</td>
<td>0.1420</td>
</tr>
<tr>
<td>Burden mean</td>
<td></td>
<td>84</td>
<td>14.0a</td>
<td>5.4</td>
<td>0.01660b</td>
<td>0.0727</td>
</tr>
</tbody>
</table>

Letters (a and b) indicate significant differences between group means (P < 0.05).

a Group burden means were calculated using total number of larvae (n) per group.

### 4. Discussion

Although several aspects of the immune response of sheep against *O. ovis* infection have been investigated, many others are not clear. It has been reported that immunization with ES products caused a decrease in *O. ovis* larval weight (Frugère et al., 2000) but the origin and nature of the involved antigens is not known. In the present study, the inflammatory local responses in the nasal and sinusal cavities were similar to that reported by Dorchies et al. (1998). According to Tabouret et al. (2003b) the immune response against *O. ovis* larvae in the frontal sinus and ethmoidal epithelium is mainly cellular, with accumulation of leucocytes (T and B lymphocytes, macrophages) and granulocytes (eosinophils, mast cells, globular leucocytes). These authors pointed out that *O. ovis* infected lambs secreted specific IgG and IgA antibodies in the nasal epithelial mucus, and this humoral response was chiefly directed against larval salivary gland antigens and not to digestive system targets. Tabouret et al. (2001) on the other hand, isolated a 28 kDa protein complex from the *O. ovis* salivary gland that showed high antigenicity. This protein complex was mainly secreted by second and third stage larvae and the antibody titers were not found correlated with larval burden. On this regard, Alcaide et al. (2005) reported a correlation between the number of *O. ovis* larvae and serum antibody levels in ELISA tests and that the stage of larval development had a significant effect on the IgG seasonal response in adult sheep, suggesting different antigenic characteristics for each larval stage. Suárez et al. (2005) observed that the number of *O. ovis* L1 larvae and serum IgM levels were positively correlated, as were L2 larvae and IgG levels, and that a seasonal reduction of IgG antibodies was associated with the end of the larval growth period or beginning of the diapausal season.
Our results demonstrate a significant but somewhat delayed effect of immunization with GMPE on serum IgG production. This delay may be related either to using FIA, or to suboptimal doses of GMPE for the immunization trial. East et al. (1992) observed the titers of antibodies against Lucilia cuprina (Wiedemann) (Diptera, Calliphoridae) in sheep were approximately the half when FIA was used, as compared to Freund’s complete adjuvant. Effects of immunization on larval establishment rate and larval development in this study were similar to those obtained by Frugère et al. (2000) who used ESP for lamb immunization. A strong response in IgG antibodies was achieved by those workers when larval ESP were used for immunization of lambs against O. ovis. Similarly, larval establishment rates were unaffected while larval size was affected by immunization. The reason for these failures are not clear. Vaccines against Lucilia are preferentially targeted on larval serine-proteases which are responsible for the breakdown of host protein. Tellam and Eisemann (2003) demonstrated that the development of L. cuprina was reduced by 84% when larvae were fed blood serum from sheep immunized with first instar antigens in vitro. An electron microscope study demonstrated that the immune response was directed toward the proteins of larval peritrophic membrane, blocking nutrient permeability and causing larval hyponeutrition. In that case, the larval basal membrane and microvilli of digestive epithelial cells were also affected. It appears that we might have observed a similar effect.

Immunization of lambs with GMP extracts delayed the progression of physiological age and decreased larval development, both in terms of burden weight and LDI. The decrease in larval development was not dramatic possibly due to a low level of antibody ingestion by the larvae. A study using L. cuprina, showed that antibodies against the peritrophic membrane can interfere with larval viability in a dose dependent fashion, so the protective efficacy of vaccination was limited by the amount of antibody available for ingestion by the larvae (Colditz et al., 2002). Likewise, nematodes that are non blood-feeders (i.e. Telaforsagia circumcincta, Trichostongylus colubriformis, and Ostertagia ostertagi in cattle) probably do not ingest sufficient levels of host immunoglobulins to seriously affect nutrient uptake (Smith et al., 2000; Newton and Meeusen, 2003). O. ovis is not considered to be a hematophagous parasite and antibody intake by larvae may not be sufficient to greatly interfere with the larval digestion/absorption processes. Moreover, it has been reported that ESP from O. ovis can cleave immunoglobulins G (Tabouret et al., 2003a), which suggests that IgG antibodies are eventually degraded by larval enzymes and sufficient IgG levels cannot be reached to block target proteins in the digestive system of O. ovis larvae. With the methods used in this study, we isolated proteins from the peritrophic membrane (exposed to the host immune system) and proteins from the intestinal mucosa (true hidden antigens). Release of ESP or peritrophic membrane antigens onto the nasal mucosa might occur during larval feeding. Three major protein bands were observed in the electrophoresis profile of GMPE (28, 38, and 55 kDa) that may be candidate antigens, but biochemical characterization of these proteins is incomplete. Some peritrophins from L. cuprina and Chrysomya bezziana (Villeneuve) (Diptera, Calliphoridae) are in this molecular weight range (Vuocolo et al., 2002; Colditz et al., 2002).

5. Conclusions

These data suggest that specific components of GMPE were able to interfere with O. ovis larval development, causing a reduced weight and delayed physiological age progression. Further work must focus on identifying and isolating the antigenic components of GMPE.

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References


