Immune response in alpacas against *Fasciola hepatica* cysteine proteinases Fas1 and Fas2*

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Abstract

A characterization of the humoral immune response of alpacas to *Fasciola hepatica* Fas1 and Fas2 antigens cysteine proteinases was performed over the course of 6 months of experimental infection. Six adult alpacas aged 1 to 2 years old received a single dose of 200 *F. hepatica* metacercariae, 2 non–infected alpacas were kept as control group. Peripheral eosinophilia in infected animals was greatly enhanced 6 weeks PI and later. A single peak of alanine aminotransferase (SGPT) was observed 4 week PI and aspartate aminotransferase (SGOT) elevated 3 weeks PI and later. All infected animals shed eggs 8 week PI and the number of flukes recovered at necropsy averaged 41 ± 4. Circulating IgG Abs against Fas1 and Fas2 were measured by ELISA. Fas2-ELISA detected the infection 10 days PI reaching to highest titer on 7-8 week PI and kept elevated until the end of infection. Fas1-ELISA detected the infection 2 weeks PI and followed the same pattern as Fas2-ELISA. Anti Fas2 IgG Abs were in higher titers and showed stronger avidity than anti Fas1 IgG Abs. In the present study we have established a *F. hepatica* experimental infection of alpacas that might be employed as a model to search for genes involved in the susceptibility to helminth infection in alpacas.

1. Introduction

Fasciola hepatica infection is a serious problem to the alpaca farming by the losses caused by the pathogen in animal productivity and mortality (Leguía and Casas, 1999). The disease is highly prevalent along the high steppes of the Andes, where the majority of alpaca farms in Peru are found. The transmission of the infection is facilitated by the heavy contamination of the pastures with the infective form, the climate condition that favors the parasite cycle and the lack of control programs against the fluke. The prevalence of F. hepatica infection in alpacas in these locations is 65% by serology with a high rate of re-infection (Neyra et al., 2002). Alpacas are highly susceptible to fasciolosis and causes mortality if not treated with flukicide (Leguía, 1991), however, there is a lack of information of the liver pathology and the immune response induced in alpacas by this trematode, even more no information is available of the genes associated to.

Production traits in alpacas include fibre characteristics, host resistance to infections, - particularly to sarcocystiosis, fasciolosis in adult animals and to Clostridium perfringens type A enterotoxemia in young animals, reproductive and metabolic traits. Fibre diameter and fleece weight are by far the major contributors to the economic value to the alpaca farming and to the associated textile industry. In addition the alpaca population is disease-ridden with parasitic infection the have a negative impact in the production of fibre and meat. This situation is jeopardising the resource by the loss of animals with fine fibre and by the increased morbidity/mortality and decreased fertility of alpacas infected with parasites. Host resistance to parasitic infections is QTL

(quantitative trait loci) determined by the action of few genes and the environment. A pivotal component to accelerate the understanding of the genetics of these traits is to establish a genomic information database for alpacas as it is already implemented for sheep and other domestic animals. Genome scanning to map QTLs involved in wool characteristics and host resistance to parasite infections are in progress in sheep.

In the present study, we report an evaluation humoral immune response against antigens Fas1 and Fas2 in alpacas experimentally infected with F. hepatica that might be used as a model to map the alpaca genes associated to resistance against helminth infections.

2. Materials and Methods

Experimental infection
Alpacas were obtained from SAIS Pachacutec, Junin and transported to Lima, where they were kept 2 months for adaptation to the housing conditions. Animals were checked for helminth infection by stool inspection and received a single dose of triclabendozole and closantel prior the experimental infection. Animals were housed in an uncovered pen and fed with dry hay and water *ad libitum*. The experimental infection was made by a single oral administration of 200 *F. hepatica* metacercariae to six adult alpacas 1 to 2 years old and 2 non–infected alpacas were kept as control group.

Parasitological studies
Stool samples were weekly collected and examined by rapid sedimentation procedure (Lumbreras et al., 1962), the parasitological inspection was performed under light microscopy. All animal were slaughtered 24 weeks PI, the liver and the gallbladder were thoroughly inspected for flukes.

Hepatic enzymes and serum proteins
Serum glutamic oxalacetic transaminase (SGOT) and glutamic piruvic transaminase (SGPT) activity was estimated using a SERA-PAK kit from Bayern (New York, USA). Enzymatic activity was expressed in International Units per Liter (IU/L). Alkaline phosphatase (ALP)-AMP, total and direct bilirubin, albumin and total proteins were measured using kits from Biosystems (Barcelona, Spain).

Enzyme-linked immunosorbent assay (ELISA)
Fas1 (1.5 μg/ml) and Fas2 (1 μg/ml) were bound to microtiter plates IMMULON®4HBX (Dynex Technologies, INC, USA) by incubation o/n at 4°C. Plates were washed five times in PBS with 0.05% Tween 20 and then incubated with 1% BSA in PBS with 0.05% Tween 20 for 1 h at 37°C. 100 μl of serum previously diluted 1/100 (Fas1) and 1/200 (for Fas2) in PBS with 0.05% Tween 20 and 2% BSA, was added to the plates and incubated for 1 h at 37°C. Plates were thoroughly washed four times as described above. 100 μl of protein A conjugated to horse radish peroxidase, previously diluted to 1/400 in PBS with 0.05% Tween 20 and 2% BSA, was added to each well and incubated for 1 h at 37°C. A color reaction was observed by incorporating TMB (3, 3', 5, 5'-tetramethyl-benzidine) into the reaction, which was stopped by adding 50 μl of 2M H_2SO_4. The optical density was measured at 450 nm (OD_{450}) in a microplate reader (Benchmark Bio-Rad).

Avidity of IgG against Fas1 and Fas2
Avidity index of IgGs against Fas1 and Fas2 was determined by the potassium isocyanate dilution method (Pullen et al., 1986). The index is estimated as the KSCN concentration needed to decrease by 50% the total bound of IgG to the antigen.

Statistical analysis
Data statistical analysis was done using GraphPad Prism™. Fas1- and Fas2-ELISA cut off values were estimated as previously described (Neyra et al., 2002).

3. Results

Experimental infection and blood cell count
All infected alpacas shed F. hepatica eggs 8 weeks PI remaining positive until the end of the experimental infection. Mature flukes were recovered from the livers of infected animals at necropsy; the parasite load averaged 41± 4 (Table 1). Infected animals haematocrit decreased 4 weeks PI onwards (Figure 1a). No difference was observed in the leukocyte population in infected and controls, however leukocytes transiently increased 4, 8 and 18 weeks PI (Figure 1b). Eosinophils increased 6 weeks PI to a maximum value on the 8th week and remained elevated during the rest of the infection (Figure 1c).
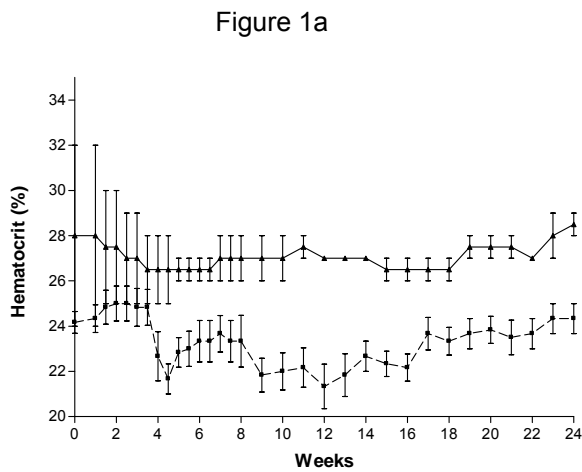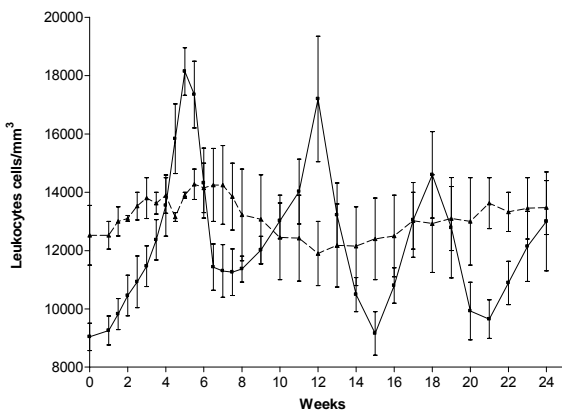
Figure 1a
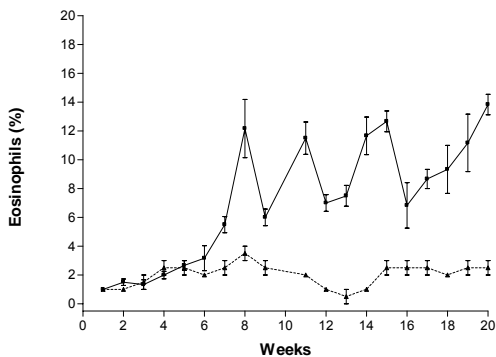

Figure 1b

Figure 1c

Figure 1. Blood cell count in F. hepatica infected alpacas and control. **1a.** Haematocrit, **1b.** Leukocytes; **1c.** Eosinophils. Infected alpacas (■) and controls (▲).

Liver Enzymes and serum proteins

A single peak in the activity of SGPT in sera of infected animals was observed 4 weeks PI (27.2 ± 6 IU/L) (Figure 2a). Serum level of SGOT reached the highest level 4 weeks PI (536.7± 82.1 IU/L) in infected animals. SGOT remained elevated in infected animals until 24 week PI (Figure 2b).
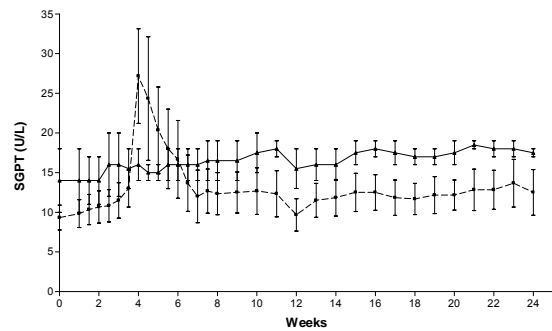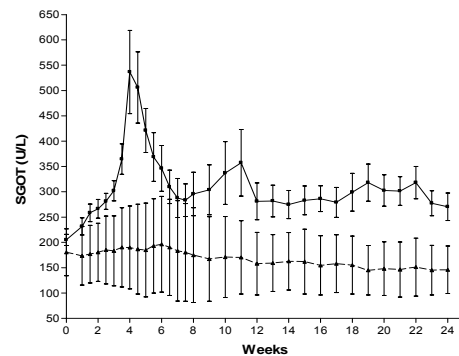
Figure 2a

Figure 2b

Figure 2. Serum level of liver enzymes in F. hepatica infected alpacas. **2a.** Glutamic piruvate transaminase (SGPT); **2b.** Glutamic oxalacetic transaminase (SGOT). Infected alpacas (■) and controls (▲).Data points represents the mean IU/L of replicates of serum samples of each animal. Infected alpacas (■) and controls (▲).

Fas1 and Fas2 ELISA

All infected alpacas were positive to Fas1-ELISA 2 weeks PI with OD_{450} 0.21 ± 0.02 (cut-off value 0.14 OD_{450}) and remained positive in ELISA to Fas1 antigen with a peak 8 weeks PI (OD_{450} 0.54 ± 0.06) (Figure 3a). A similar pattern was observed with Fas2-ELISA, infected animals were positive 10 days PI with O_{450} 0.184 ± 0.018 (cut-off value 0.14 OD_{450}), reached a peak 8 weeks PI (O_{450} 0.624 ± 0.07) and remained elevated until week 24 PI (Figure 3b).
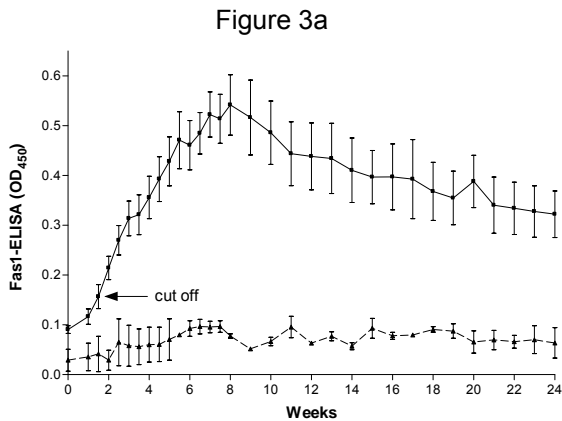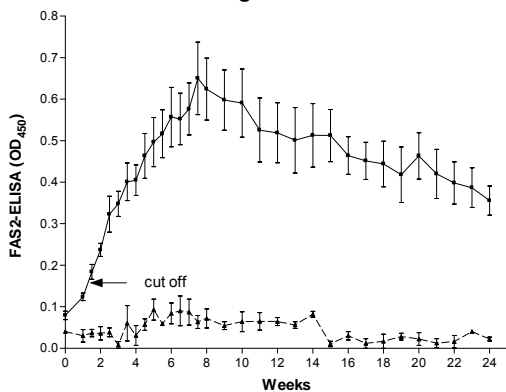
Figure 3a

Figure 3b

Figure 3. IgG circulating antibodies against Fas1 and Fas2 detected by ELISA. F. hepatica experimentally infected alpacas (n=6) and controls (n=3) were evaluated by ELISA with Fas1 **3a.** and Fas2 **3b**. Numbers and arrows represent the cut-off values set as OD_{450} for the serological assays with the corresponding antigens. Data points represents the mean $OD_{450} \pm$ SME of replicates of serum samples of each animal. Infected alpacas (■) and controls (▲).

IgG Ab avidity against Fas1 and Fas2
Three infected alpacas and two controls were used to estimate the avidity index of circulating IgG Abs against Fas1 and Fas2 in sera collected 4, 8 and 24 weeks PI. Anti Fas2 IgG Abs had higher avidity index (76.43 $\pm$ 1.84) than anti Fas1 IgGs (49.7 $\pm$ 0.3) at 2N KSCN. The avidity index did not change during the course of the infection for both antigens (Figure 4).
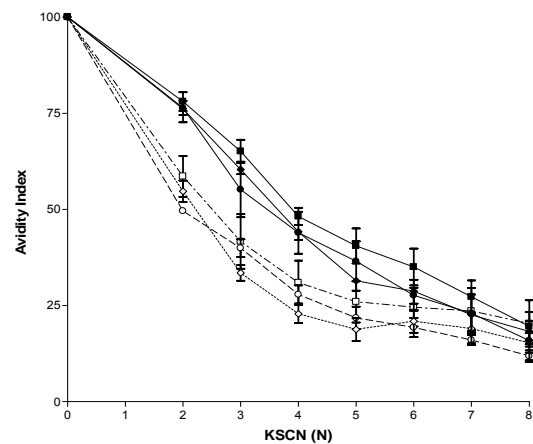
Figure 4

Figure 4. Avidity index of circulating IgG antibodies against: **Fas1**, 4 weeks PI (○), 8 weeks PI (◊), 24 weeks PI (□); **Fas2**, 4 weeks PI (●), 8 weeks PI (♦), 24 weeks PI (■). Each point represents the mean $OD_{450} \pm$ SME of replicates of serum samples of three F. hepatica infected alpacas.

4. Discussion

In this work, we established an experimental infection of alpacas with F. hepatica. The primary infection is similar to those observed in other species susceptible to F. hepatica infection: mature flukes shed eggs by the 8 week PI and adult worms were recovered from the bile ducts at necropsy (Chauvin et al., 1995; Clery et al., 1996; Ruiz et al., 2003). The parasite reached the liver by the 4 week and produced damage in the liver parenchyma elevating both liver enzymes SGPT and SGOT. No change in bilirubin and alkaline phosphatase suggested that the infection did not produce biliary obstruction. SGOT remained elevated while SGPT declined during the course of the infection. SGOT is characteristic of the liver damage caused by the parasite in alpacas as previously observed in cattle (Leclipeteux et al., 1998). The infection caused anaemia, which is considered an important factor of the host morbidity and mortality by fluke infections (Behm & Sangster, 1999). However, no change in protein, albumin and body weight was observed in infected animals that may reflect that the parasite burden is probably low compared to the number of flukes found in natural infected animals, where weight loss, emaciation and mortality are commonly observed in untreated animals (Leguia and Casas, 1999). A transient elevation of leukocytes was

observed in infected animals in different periods of the infection, which may be related to the pathogenicity of the fluke infection.

As observed in other species (reviewed in Behm & Sangster, 1999), the infection caused a dramatic increase in peripheral eosinophilia that initiated in the late acute phase, 6 weeks PI, and expanded through the chronic phase. No correlation was found between eosinophilia and IgG titers against Fas1 and Fas2, being peripheral eosinophilia observed after the elevation of IgG Abs level against the parasite antigens. We observed more than one peak of eosinophilia during the infection; similar finding was reported in sheep (Chauvin et al., 1995). It is arguably if this is due to migration of eosinophils from blood to the liver or exacerbation of the immune response of the host against the parasite re-invasion of a bile duct. Eosinophilia is a characteristic feature of the immunological response against helminth infection (Janeway et al., 2001). Eosinophils are involved in the antibody dependent cell-mediated cytotoxicity against the related trematode *Schistosoma mansoni.* Eosinophils and other cytotoxic cells appear to participate in the antibody mediated killing of juvenile forms of F. hepatica in a variety of species (Piedrafita et al., 2001; Mulcahy et al, 1999). It is suggested that is mediated by a cellular Th2 like response in cows (Bossaert et al., 2000) and model animals such as mice and rats (Milbourne & Howell, 1997), eosinophilia is also a response to the fluke in alpacas that extended to the chronic phase of infection, however the role of T helper cells in the immune regulation against infection is not characterized in these camelids.

F. hepatica infected alpacas elicited circulating IgGs against cysteine proteinases Fas1 and Fas2 as previously observed in natural infected alpacas (Neyra et al., 2002). Highest levels of anti Fas1 and anti Fas2 IgG Abs were observed 8 weeks PI coincidental with the beginning of egg shedding by infected animals. IgG titers remained elevated during the experimental infection, suggesting a permanent immune stimulus caused by these cysteine proteinases that are released to the fluke gut lumen and emptied by regurgitation by the adult worm while residing in the bile ducts (Dalton et al., 2003). As observed in other species, infected alpacas elicited a stronger humoral response with higher circulating titers and stronger antibody avidity against Fas2 compared to Fas1 (Strauss et al., 1999; Cordova et al., 1999). In helminth infection of other species, a significant change in avidity of IgG Abs was strongly associated to the phase of the infection (Viana et al., 2001). No change in the avidity index of IgG Abs against Fas1 and Fas2 was observed in infected animals during the course of infection (Figure 4). This observation suggests either reinfection, these animals were formerly raised in endemic areas for fasciolosis so animals had a cured previous infection, or a particular mechanism of antibody production in camelids, considering the IgG Abs differ from all known IgGs of other species (Hamers-Casterman et al., 1993). It is reported that Fas2 and Fas1 share common epitopes and certain intrinsic epitopes in Fas2, not present in Fas1, endows this antigen with a better performance characteristics in ELISA to detect F. hepatica infection (Cordova et al., 1997, 1999). Serodiagnosis using Fas2 detected the infection earlier than Fas1 in all infected animals. Our results suggest that Fas2 and Fas1 cysteine proteinases are major immunodominant antigens in F. hepatica infection in alpacas.

In conclusion, F. hepatica infected alpacas elicit a strong immune response against the parasite characterized by an increase in eosinophils, a transient elevation of leukocytes and elevation of circulating IgG Abs against two major parasite antigens Fas1 and Fas2. Anti Fas2 IgG Abs are in high titers, are detected as early as 10 day PI and display strong avidity. We have characterized a helminth infection in alpacas in ordr to develop the basis for an approach to map the genes associated to resistance to fascioliasis in alpacas.

Acknowledgements

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Table 1. Experimental infection of alpacas with F. hepatica.

Alpaca Code	Sex	Age (years)	Total number of flukes	% Recovered Flukes*
1011	female	1.6	36	18
500	female	1.6	44	22
2207	male	1.6	44	22
176	male	1.6	42	21
991349	male	2	45	22.5
9881	male	2	36	18
MA996	male	2	0	0
PP	female	1.4	0	0

*Alpacas received a single dose of 200 metacercariae.