Comparison of humoral response in sheep to Fasciola hepatica and Fasciola gigantica experimental infection

W. Zhang, Emmanuelle Moreau, W. Huang, Alain Chauvin

COMPARISON OF HUMORAL RESPONSE IN SHEEP TO *FASCIOLA HEPATICA* AND *FASCIOLA GIGANTICA* EXPERIMENTAL INFECTION

ZHANG W.*, **, MOREAU E.**, HUANG W.* & CHAUVIN A.**

Summary:

Humoral response of sheep to *F. gigantica* was compared with the well known humoral response to *F. hepatica*, in order to explain the difference of susceptibility of sheep to these two parasites. In this work, a lesser susceptibility of sheep to *F. gigantica* than to *F. hepatica* infection was confirmed. Humoral response to *F. hepatica* infection is similar to that previously described by several authors. IgG level of *F. gigantica* infected sheep increased from week 2 post-infection (2WPI) and displayed a peak at 13WPI. *F. gigantica* excretory-secretory products (FgESP) analyzed by SDS-PAGE showed at least 31 bands from 12.0 to 127.6 kDa in FgESP. Western blot indicated that *F. gigantica* infected sheep sera recognized, in FgESP, at least 30 antigens from 7.8 to 119.2 kDa of which 12 major bands recognized after 0WPI. In FhESP and FgESP, *F. hepatica* infected sheep serum reacted only with the lower molecular mass antigens, while *F. gigantica* infected sheep serum reacted with the lower and the higher molecular mass antigens. These differences of antigenic recognition might be associated with the difference of susceptibility of sheep. Further investigation must be done to study the mechanism of resistance between the sheep infected with *F. hepatica* or *F. gigantica*.

KEY WORDS : humoral response, sheep, *Fasciola hepatica*, *Fasciola gigantica*, SDS-PAGE, ELISA, Western blot.

Résumé : COMPARAISON DE LA RÉPONSE HUMORALE DE MOUTONS INFESTÉS EXPÉRIMENTALEMENT PAR *FASCIOLA HEPATICA* OU PAR *FASCIOLA GIGANTICA*

La réponse humorale du Mouton induite par F. gigantica *a été comparée à celle induite par* F. hepatica, *bien connue, afin d'expliquer les différences de sensibilité du Mouton à ces deux parasites. Dans cette étude, la moindre sensibilité du Mouton à* F. gigantica *par rapport à* F. hepatica *a été confirmée. La réponse humorale à* F. hepatica *est similaire à celle décrite précédemment par de nombreux auteurs. La quantité d'IgG des moutons infestés par* F. gigantica *augmente dès la 2ème semaine après infestation (SPI2) et atteint un pic en SPI13. L'analyse des produits d'excrétion-sécrétion de* F. gigantica *(PESFg) par SDS-PAGE a révélé au moins 31 bandes de 12.0 à 127.6 kDa. Les Western blot indiquent que les moutons infestés par* F. gigantica *reconnaissent dans les PESFg au moins 30 antigènes de 7.8 à 119.2 kDa dont 12 bandes majeures détectées après SPI0. Dans les deux PES, les sérums des moutons infestés par* F. hepatica *réagissent uniquement avec les antigènes majeurs de bas poids moléculaires alors que ceux des moutons infestés par* F. gigantica *réagissent avec les antigènes majeurs de bas et de hauts poids moléculaires. Ces différences de reconnaissance antigénique pourraient être associées aux différences de sensibilité des moutons. D'autres investigations seraient nécessaires pour étudier les mécanismes de résistance des moutons infestés par* F. hepatica *ou par* F. gigantica.

MOTS CLÉS : *réponse humorale, mouton,* Fasciola hepatica, Fasciola gigantica, *SDS-PAGE, ELISA, Western blot.*

INTRODUCTION

Fasciolosis caused by two species of the genus *Fasciola*, *F. hepatica* and *F. gigantica*, is a parasitic disease of economical importance in ruminants. *F. hepatica* usually infects sheep and cattle, while *F. gigantica* infection often appears in cattle and buffaloes in the tropical regions. In China, both *F. hepatica* and *F. gigantica* infections are frequently found in sheep, cattle or buffaloes. Sheep, although very susceptible to *F. hepatica* infection with no resistance to reinfection (Boyce *et al.*, 1987), are less susceptible to *F. gigantica* infection with even a high resistance in certain breeds (Roberts *et al.*, 1997). A better knowledge of the mechanism of resistance or susceptibility to *Fasciola* sp. infection in animals would be helpful to better control this parasitic disease. In different hosts (sheep, goat, cattle, rat), the immune response against *F. hepatica* is characterised by an early increase of antibody level (Oldham, 1985; Poitou *et al.*, 1993; Chauvin *et al.*, 1995; Clery *et al.*, 1996; Martinez-Moreno *et al.*, 1997; Chen *et al.*, 2000). Several antigens were described in *F. hepatica* excretory-secretory products (FhESP) (reviewed by Spithill *et al.*, 1999b). The humoral immune response to *F. gigantica* infection was less studied but seemed to be very similar (Guobadia & Fag-

* College of Animal Science and Technology, Guangxi University, 530005 Nanning China.
** UMR INRA/ENVN Interactions Hôte-Parasite-Milieu, École Nationale Vétérinaire de Nantes, BP 40706, F-44307 Nantes Cedex 03, France.
Correspondence: Emmanuelle Moreau.
Tel.: 33 (0)2 40 68 78 58 – Fax: 33 (0)2 40 68 77 51.
E-mail: moreau@vet-nantes.fr

bemi, 1995; Mbuh & Fagbemi, 1996; Gupta & Yadav, 1995). Hansen *et al.*, (1999) analysed the immune response to *F. gigantica* of a resistant (Indonesian thin tail) and a susceptible (Merino) breed of sheep, but no investigation was done to compare the humoral response of a breed of sheep to *F. hepatica* and *F. gigantica* to explain the difference of suceptibility of this breed to both infections. For this purpose, we investigated the kinetics of the humoral immune response of sheep infected with *F. gigantica*, analysed the antigens of excretory-secretory products of *F. gigantica* (FgESP) and *F. hepatica* (FhESP) recognized during the infection and compared this response with the one developed by sheep infected with *F. hepatica*.

MATERIALS AND METHODS

F. HEPATICA AND *F. GIGANTICA* METACERCARIAE ORIGINS

F. hepatica and *F. gigantica* metacercariae were obtained from *Lymnea truncatula* and *Lymnea auricularia* snails respectively using exactly the same method. The six-month-old metacercariae were maintained at 4° C until use. *F. hepatica* metacercariae were kindly provided by P. Sibille (INRA, Tours, France) and *F. gigantica* metacercariae originated from China (College of Animal Science and Technology, Guangxi University, Nanning, China).

SHEEP EXPERIMENTAL INFECTION

15 two-year-old "Belle Islois" male sheep were divided into three groups of five animals. Group C served as control. Groups H and G were infected *per os* with a gelatin capsule containing 250 *F. hepatica* or *F. gigantica* metacercariae respectively. The animals were born and maintained in a sheep-fold. Feed and water were available *ad libitum*. All animals were bled weekly from week 0 post-infection (0WPI) to 15WPI and serum samples were collected. Necropsy was performed at 15WPI to recover and count the flukes from the liver of each infected animal.

PRODUCTION OF *F. HEPATICA* AND *F. GIGANTICA* EXCRETORY-SECRETORY PRODUCTS (FHESP AND FGESP)

Adults *F. hepatica* or *F. gigantica* were removed from the bile duct of *F. hepatica* infected sheep or *F. gigantica* infected buffaloes respectively. The flukes were washed several times in phosphate-buffered saline (PBS) pH 7.2, and then incubated in PBS at room temperature for three hours. The supernatant fluids were filtered through a 20 µm nylon filter to remove the eggs, centrifuged at 3,000 *g* for 15 min to remove particulate material and filtered through a 0.45 µm cellulose acetate filter. The protein content was determined by the Bicinchoninic Acid (BCA) method (Pierce, USA).

DETERMINATION OF SHEEP ANTIBODY LEVELS DURING INFECTIONS BY ELISA

The antibody levels to FhESP and to FgESP of each animal serum were determined using an ELISA previously described by Chauvin *et al.* (1995) with some modifications. Microplates (Microwell Nunc) were coated with FhESP or FgESP (2.5 µg protein/ml) in 0.1 M carbonate buffer pH 9.6 at 37° C for one hour, then at 4° C overnight. After three washes with PBS 0.1 % Tween 20 (PBST), 150 µl of gelatin (Bio-rad, USA) solution (2 % w/v in 0.1 M carbonate buffer pH 9.6) were added to each well and incubated for 30 min at 37° C. Standard positive and negative sera and test sera (sera from sheep of groups C, H or G, diluted at 1/100 in PBST) were added in duplicate to the wells (100 µl per well) and incubated for one hour at 37° C. After three washes in PBST, 100 µl of anti-sheep IgG peroxidase conjugate (Sigma, USA; dilution of 1/2000 in PBST) were added to each well and incubated for one hour at 37° C. After three washes in PBST and PBS, 100 µl of ABTS (2, 24-azino-bio 3-ethylbenz-thiazoline-6-sulfonic acid) substrate (1.1 mg/ml, Sigma, USA) in citrate buffer 0.1 M pH 4 with 0.13 µl/ml H_2O_2 30 % were added to each well. After incubation for one hour at 37° C, and then for 15 min at 4° C, the optical densities (O.D.) were read at 405 nm using an ELISA automate (MRX Microplate reader, USA).

Mean of O.D. (mO.D.) of the five sheeps for each group and for each ESP was calculated weekly (mO.D. at 0WPI, mO.D. at 1 WPI, mO.D. at 2 WPI, ...mO.D. at nWPI). Then, ΔO.D. were calculated weekly as follow:

$$\Delta O.D. = mO.D. \text{ at } nWPI - mO.D. \text{ at } 0\,WPI$$

COMPARISON OF FHESP AND FGESP BY SDS-PAGE

Comparison of the two ESP was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mighty Small II SE250 (Hoefer scientific instruments, USA). Samples containing 15 µl (5 µg) FhESP or FgESP and 5 µl sample buffer (0.25 M Tris-HCl (pH 6.8 at 25° C), 8 % (w/v) SDS, 40 % glycerol, 0.04 % (w/v) bromophenol blue and 20 % mercaptoethanol) were boiled for two minutes and cooled immediately on ice. Samples were applied to a 4 % stacking gel and resolved through a 12 % resolving gel at 4° C. After electrophoresis, gels were fixed and stained using the Silver Staining procedure.

COMPARISON OF ANTIGENS CONTAINED IN FHESP AND FGESP BY WESTERN BLOT

Western blot analysis of IgG response of infected animals was carried out using the procedure previously

described by Chauvin *et al.* (1995). FhESP or FgESP were separated using SDS-PAGE. Proteins were then transferred electrophoretically on to nitrocellulose of 0.45 µm pore size (Bio-rad, USA) with a semi-dry transfer unit (Semi-phor TE70, Amersham Bioscience, USA). Nitrocellulose (NC) sheets were then saturated with 10 % skimmed milk powder in 0.1 M Tris-buffered saline (pH 7.6) with 0.1 % Tween (TBST). NC strips were incubated for 45 minutes with each test serum (individual sheep serum from groups C, H or G sheep) diluted at 1/100 in TBST/M (TBST and 5 % skimmed milk powder). After three washes in TBST, NC strips were successively incubated with biotin-conjugated mouse anti-sheep IgG (Sigma, USA; dilution of 1/1,000 in TBST/M) for 45 minutes and Avidin and biotinylated alkaline phosphatase (Vectastain ABC-AP kits, Vector Laboratories Inc., U.S.A.; dilution of 1/5 in TBST) for 45 minutes separated by three washes in TBST. After three more washes in TBST and distilled water, immunoreactive antigens were revealed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate system (Sigma, USA).

For each infected sheep (by *F. hepatica* or by *F. gigantica*), the humoral response to FhESP and FgESP was studied by ELISA and by western blot to investigate cross reactions between the antigens of the two parasites and to determine if the differences of humoral responses observed were due to the difference of *F. hepatica* or *F. gigantica* infection or to the differences of antigens of the ESP.

Calculation of molecular masses

The molecular masses (*m*) of the bands in SDS-PAGE or Western blot were calculated by comparison of their migration with the standard molecular weight markers (Low Molecular Weight Standard, Amersham biotech for SDS-PAGE, or Kaleidoscope prestained standards, BIO-RAD, USA for Western blot). The software *BIO-PROFIL Bio-1D++ Windows application Version 10.02* was used to detect the bands and calculate their molecular masses. Mean *m* and standard error (SEM) of means *m* were determined on different migrations.

Statistical analysis

The numbers of flukes recovered from sheep (groups H and G), the antibody titer to FhESP or FgESP of infected and control sheep and of *F. hepatica* and *F. gigantica* infected sheep were compared by the non-parametric Mann-Whitney test. The antibody quantities produced against FhESP or FgESP in each group of infected sheep were also compared weekly using the non-parametric Wilcoxon test. All tests were done using the software *Statview 5.0.*

RESULTS

Necropsy

The mean fluke recovery was respectively 18.1 % (± 4.8) and 5.4 % (± 2.1) in *F. hepatica* and *F. gigantica* infected sheep, a statistically significant difference ($p < 0.05$).

Comparison of humoral response during the *F. hepatica* and *F. gigantica* infections by ELISA

Sera collected weekly from the 15 sheep during the course of the 15 weeks infection period were analysed by ELISA with both ESP (FhESP and FgESP separately). Kinetics of humoral response are similar in both infected group (Fig. 1). Anti FhESP or FgESP-IgG levels increased from 2WPI in the two groups of infected sheep and the difference between control group and infected groups was significant from 2WPI until the end of infection. The IgG responses to FhESP or FgESP displayed a peak at 10 WPI in group H and at 13WPI in group G.

In groups H and G, the humoral responses to FgESP were significantly stronger than the humoral responses to FhESP from 4WPI and 3WPI respectively (Fig. 1). The anti FgESP-IgG level was significantly greater in group G than in group H from 3WPI but there are no differences between groups H and G for the anti-FhESP-IgG level (Fig. 1).

Comparison of FhESP and FgESP by SDS-PAGE

SDS-PAGE analysis of FhESP and FgESP is shown in Figure 2. FhESP analysis revealed at least 25 polypeptide bands with molecular masses ranging from 13.4 to 135.1 kDa with three clusters of intense bands (52.3 and 55.3 kDa; 26.1, 27.3 and 28.5 kDa; 12.9, 13.6 and 15.1 kDa) whereas at least 31 polypeptide bands with a molecular masses ranging from 12.0 to 127.6 kDa were observed with FgESP. In this case, two clusters of intense bands (bands between 27.1 and 31.9 kDa and a band at 16.1 kDa) were present.

Comparison of FhESP and FgESP antigens by Westen blot

Antigens contained in FhESP and FgESP and kinetics of recognition of these antigens during the infection were analysed by Western blot. Sera collected weekly from the 10 infected sheeps during the course of the 15 weeks infection period were tested with both ESP. Figure 3 represents western blots obtained with sera of one representative sheep of group H (Fig. 3a and 3c) and one representative sheep of group G (Fig. 3b and 3d).

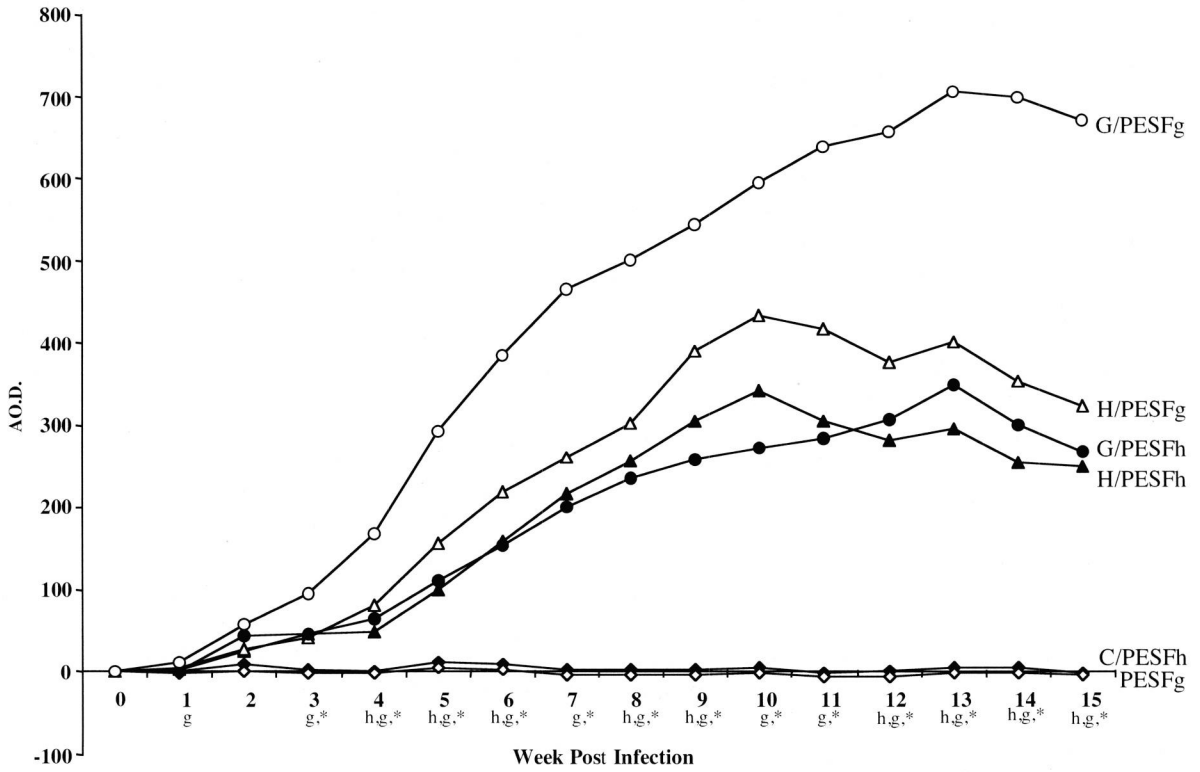

Fig. 1. – Kinetic studies of FhESP or FgESP-specific IgG response in *F. hepatica* or *F. gigantica* infected and control sheep (control sheep (group C) response towards FgESP ◇ or FhESP ♦, *F. hepatica* infected sheep (group H) response toward FgESP Δ or FhESP ▲, *F. gigantica* infected sheep (group G) response toward FgESP O or FhESP ●). h and g indicate statistical differences ($p < 0.05$) between the antibody titers to FhESP and to FgESP in group H and in group G respectively. * indicates statistical differences ($p < 0.05$) of the antibody titers to FgESP between group H and group G.

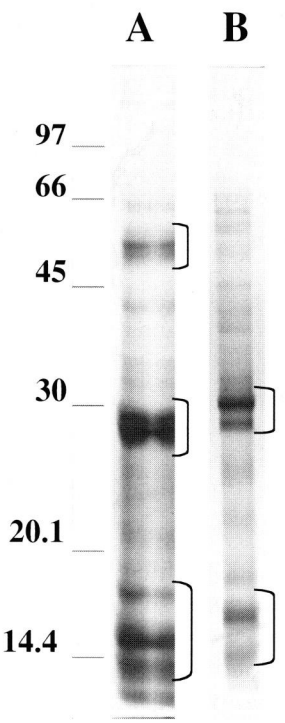

Fig. 2. – SDS-PAGE comparison of FhESP and FgESP. The horizontal bars on the left indicate the molecular mass of standard markers (kDa). Lane A: FhESP; lane B: FgESP. The vertical bars on the right of lane A and lane B indicate the clusters of the intense bands.

Kinetics of antigens recognition during *F. hepatica* and *F. gigantica* infections were followed as the evolution of staining intensity of bands. The staining intensity of most bands increased up to 10 or 12WPI and then decreased. The recognition of the major antigens (antigens of FhESP recognized by all five *F. hepatica* infected sheep or antigens of FgESP recognized by all five *F. gigantica* infected sheep) that appeared after infection in FhESP began at 5WPI for *F. hepatica* infected sheep sera and between 3WPI and 10WPI for *F. gigantica* infected sheep sera. In FgESP, the recognition of the bands that appeared after infection began between 2WPI and 5WPI for *F. gigantica* infected sheep serum and between 4WPI and 7WPI for *F. hepatica* infected sheep serum.

In FhESP *F. hepatica* infected sheep sera recognized 24 antigens all along the infection, with 12 major bands (antigens recognized by all five *F. hepatica* infected sheep) indicated in Figure 3a. Six major bands of 14.9, 17.1, 18.3, 19.3, 21.6 and 31.9 kDa were recognized after 0WPI by *F. hepatica* infected sheep sera and were not recognized by control sheep sera. *F. gigantica* infected sheep sera recognized 30 antigens in FhESP with five major bands recognized by all five *F. gigantica* infected sera, indicated in Figure 3b.

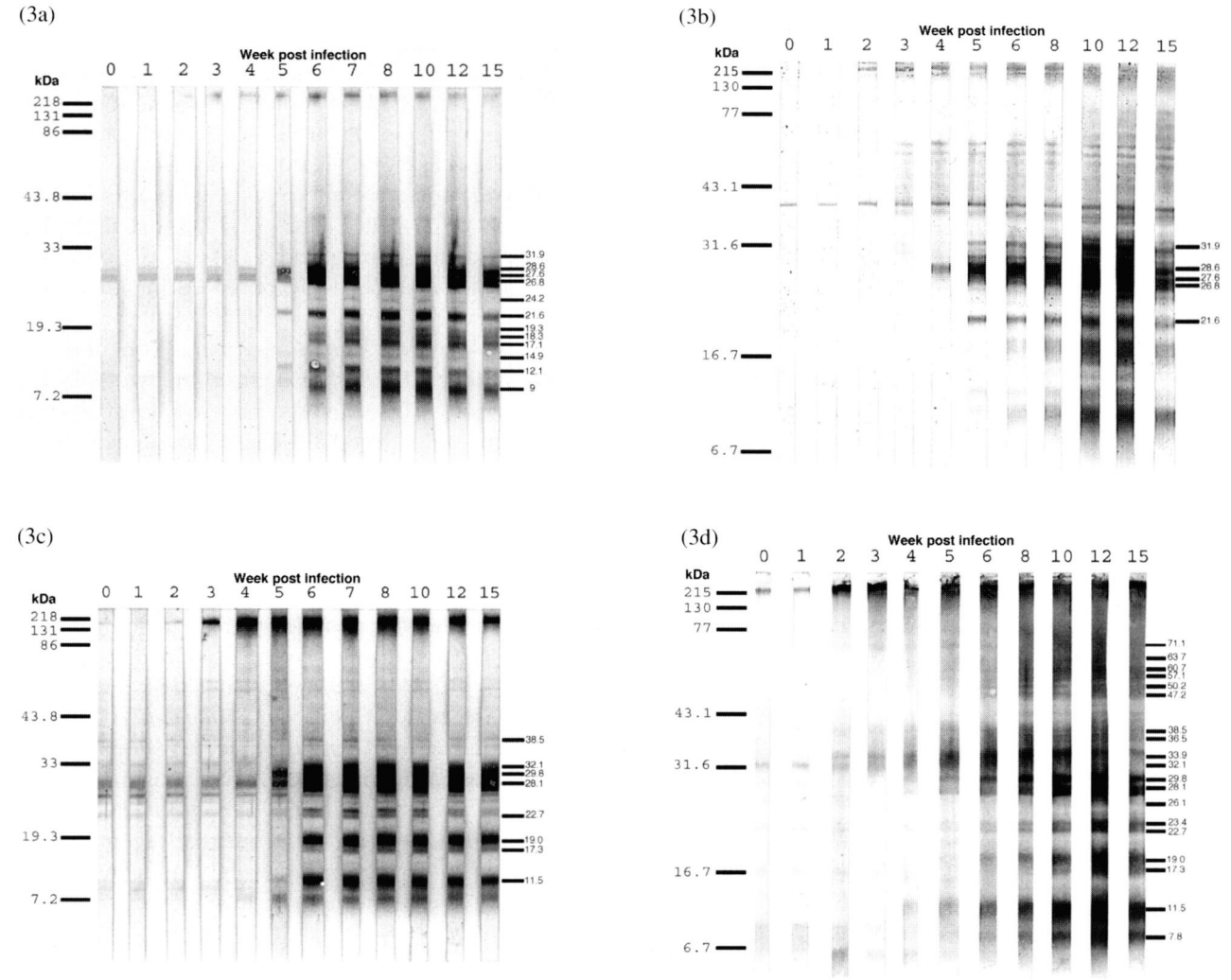

Fig. 3. – Western blot analysis of IgG response to FhESP of a representative sheep infected with *F. hepatica* (3a), or with *F. gigantica* (3b), and IgG response to FgESP of a representative sheep infected with *F. hepatica* (3c) or with *F. gigantica* (3d). The bars on the left indicate the marker molecular mass (kDa); the bars on the right indicate the molecular masses of the major bands.

In FgESP 30 antigens from 7.8 to 119.2 kDa were recognized by *F. gigantica* infected sheep sera all along the infection, with 18 major bands (recognized by all five *F. gigantica* infected sheep) indicated in Figure 3c. Twelve major bands of 11.5, 19.0, 23.4, 29.8, 33.9, 36.5, 38.5, 47.5, 50.6, 57.9, 62.5 and 71.2 kDa were recognized after 0WPI by *F. gigantica* infected sheep sera and were not recognized by control sheep sera. Twenty six immunoreactive bands were recognized in FgESP by *F. hepatica* infected sheep sera: eight major antigens were recognized by all five *F. hepatica* infected sheep sera indicated in Figure 3d.

Strong reactivities between FhESP and FgESP antigens were observed. Most of the major antigens in FhESP and FgESP were commonly recognized by the two groups of infected sheep sera. However, *F. hepatica* infected sheep sera reacted more strongly with most of the lower molecular mass antigens (from 9.0 to 31.9 kDa in FhESP, from 11.5 to 36.6 kDa in FgESP (Fig. 3a and 3c)) than *F. gigantica* infected sheep sera. *F. gigantica* infected sheep sera mostly recognized the higher molecular masses antigens (from 21.6 to 40.2 kDa in FhESP, from 7.8 to 71.2 kDa in FgESP (Fig. 3b and 3d)). Some bands in FhESP and in FgESP were recognized only by *F. gigantica* infected sheep serum, but all of these bands were minor antigens (antigens not recognized by all five *F. hepatica* or *F. gigantica* infected sheep).

DICUSSION

In our present study, more flukes were recovered from *F. hepatica* infected sheep than from *F. gigantica* infected sheep. This could not be due to the difference of viability between *F. hepatica* and *F. gigan-*

tica metacercariae because they were produced exactly with the same method and were six months old. This result was similar to those of other works in different sheep breeds (reviewed by Spithill *et al.*, 1999a). This could suggested that "Belle Islois" sheep are more susceptible to *F. hepatica* infection than to *F. gigantica*. This observation should be confirmed by a study of the reinfection with these two parasite species in sheep.

The anti-FhESP and anti-FgESP IgG responses of *F. hepatica* infected sheep were increased as soon as 2WPI and reached a peak in 10WPI, as previously described in sheep by many authors (Santiago & Hillyer, 1988; Ruiz-Navarrete *et al.*, 1993; Chauvin *et al.*, 1995; Rodriguez-Pérez & Hillyer, 1995; Ferre *et al.*, 1997). Kinetics of humoral response of *F. gigantica* infected sheep was similar, but with a delayed peak at 13WPI, as described by Guobadia & Fagbemi (1995). This could be explained by a longer migration of *F. gigantica* than *F. hepatica* in the liver parenchyma, an hypothesis supported by the results of eggs recovery in feces at 10 or 11WPI for *F. hepatica* and at 13WPI for *F. gigantica* (data not shown).

For FhESP, proteins and antigens revealed by SDS-PAGE and western blot respectively are similar to those previously described in *F. hepatica* infected sheep by Chauvin *et al.* (1995) and Moreau *et al.* (1998) when using exactly the same techniques. The absence of recognition of immunoreactive major proteins above 31.9 kDa was the only noticeable difference.

SDS-PAGE of FgESP revealed at least 31 bands from 12.0 to 127.6 kDa with two clusters of prominent bands between 27.1 and 31.9 kDa and between 11.9 and 16.1 kDa. Huang *et al.* (1997), Intapan *et al.* (1998) and Maleewong *et al.* (1999) have previously described this profile, with small differences probably due to FgESP preparation and staining procedure. The bands of close molecular masses in FhESP and FgESP are probably identical bands like bands (27.3 and 28.5 kDa in FhESP and 27.1 and 28.9 kDa in FgESP, respectively). This must be confirmed by other investigations using 2D electrophoresis or protein sequencing.

F. gigantica infected sheep sera recognized at least 30 antigens in FgESP with 18 major bands from 7.8 to 71.1 kDa, of which 12 bands were recognized already after only 0WPI. The recognition of antigens in FhESP at 0WPI was previously described by Chauvin *et al.* (1995). This could be explained by several hypothesis: cross reactivity between antigens of *Fasciola* sp. and antigens of bacteria or viruses that naturally infected our sheep, molecules in ESP that fix non specifically sheep IgG... All *F. gigantica* infected animals recognized the bands of 28.1 and 32.1 kDa with an increasing staining density from 5 or 6WPI as shown for the bands of 27.6 and 28.6 kDa in FhESP. These bands could correspond to the cathepsin L proteinases or GST and the band of 11.5 kDa could correspond to the fatty acid binding protein (FABP) described by Estuningsih *et al.* (1997) in FgESP. Guobadia & Fagbemi (1995) described that *F. gigantica* infected sheep sera recognized four bands of 17, 21, 57 and 69 kDa in FgESP. The three last could correspond to the 19.0, 63.7 and 71.2 kDa bands respectively observed in our experiment.

By ELISA, we have shown that sera from the two groups of infected sheep recognized the two ESP with similar kinetics. This suggests cross-reactions between the antigens of the two ESP. These cross-reactions are clearly evidenced by western blot: both infected sheep serum recognized common antigens in FhESP or FgESP. These strong cross-reactions indicate that both FhESP and FgESP could be used for a common diagnosis of *Fasciola* sp. infection.

However, differences of antigenic recognition between the two infected groups were also observed. The higher molecular mass antigens in FhESP and FgESP were recognized strongly by *F. gigantica* infected sheep but faintly by *F. hepatica* infected sheep. On the contrary, the lower molecular mass antigens in both ESP reacted stronglier with *F. hepatica* infected sheep. Several authors described a sequential recognition of higher then lower molecular weight antigens during *F. hepatica* infection in "Vendean" sheep or cattle (Chauvin *et al.*, 1995; Itagaki *et al.*, 1995). Hoyle *et al.* (2003) demonstrated that exposure to very early juvenile flukes is able to stimulate significant functional resistance to reinfection in the bovine host and they observed that the immune response was dominant against the higher molecular weight proteins (52, 55, 68-70 and 82-96 kDa) during the early invasive stages of infection. So, the recognition of the higher molecular mass antigens by *F. gigantica* infected sheep could be associated with the resistance observed during *F. gigantica* infection in "Belle Ilois" sheep. Further investigations about antigens of *F. hepatica* and *F. gigantica* are needed to better explain the differences of susceptibility between the two parasitic infection in sheep.

ACKNOWLEDGEMENTS

This work was supported by the "Programme de Recherches Avancées" (PRA B99-04) and National Natural Science Foundation of China (30260082). The finances and the technical assistance from laboratory staff are acknowledged. We also are grateful to Laurence Malandrin for her critical comments of this manuscript.

REFERENCES

BOYCE W.M., COURTNEY C.H. & LOGGINS P.E. Resistance to experimental infection with *Fasciola hepatica* in exotic and domestic breeds of sheep. *International Journal for Parasitology*, 1987, *17*, 1233-1237.

CHAUVIN A., BOUVET G. & BOULARD C. Humoral and cellular immune responses to *Fasciola hepatica* experimental primary and secondary infection in sheep. *International Journal for Parasitology*, 1995, *25*, 1227-1241.

CHEN L., DAUGSCHIES A., WANG B.Y. & MAO X.Z. Blood eicosanoids and immune indices during fasciolosis in water buffaloes. *Parasitology International*, 2000, *49*, 273-278

CLERY D., TORGERSON P. & MULCAHY G. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. *Veterinary Parasitology*, 1996, *62*, 71-82.

ESTUNINGSIH S.E., SMOOKER P.M., WIEDOSARI E., WIDJAJANTI S., VAIANO S., PARTOUTOMO S. & SPITHILL T.W. Evaluation of antigens of *Fasciola gigantica* as vaccines against tropical fasciolosis in cattle. *International Journal for Parasitology*, 1997, *27*, 1419-1428.

FERRE I., ORGEGA-MORA L.M. & ROJO-VÁZQUEZ F.A. Serum and bile antibody responses (IgG and IgA) during subclinical *Fasciola hepatica* infection in sheep. *Veterinary Parasitology*, 1997, *68*, 261-267.

GUOBADIA E.E. & FAGBEMI B.O. Time-course analysis of antibody response by EITB and ELISA before and after chemotherapy in sheep infected with *Fasciola gigantica*. *Veterinary Parasitology*, 1995, *58*, 247-253.

GUPTA S.C. & YADAV S.C. Antibody response of rabbit to enhanced doses of *Fasciola gigantica* experimental infection. *Journal of Veterinary Parasitology*, 1995, *9*, 73-77.

HANSEN D.S., CLERY D.G., ESTUNINGSIH S.E., WIDJAJANTI S., PARTOUTOMO S. & SPITHILL T.W. Immune responses in Indonesian thin tail and Merino sheep during a primary infection with *Fasciola gigantica*: lack of a specific IgG_2 antibody response is associated with increased resistance to infection in Indonesian sheep. *International Journal for Parasitology*, 1999, *29*, 1027-1035.

HOYLE D.V., DALTON J.P., CHASE-TOPPING M. & TAYLOR D.W. Pre-exposure of cattle to drug-abbreviated *Fasciola hepatica* infections: the effect upon subsequent challenge infection and the early immune response. *Veterinary Parasitology*, 2003, *111*, 65-82.

HUANG W.Y., ZHANG W.Y. & TIAN E. Analysis of *Fasciola gigantica* excretory-secreting antigens. *Journal of Guangxi Agricultural University*, 1997, *16*, 115-124.

INTAPAN P.M., MALEEWONG W., WONGKHAM C., TOMANAKARN K., IEAMVITEEVANICH K., PIPITGOOL V. & SUKOLAPONG V. Excretory-secretory antigenic components of adult *Fasciola gigantica* recognized by infected human serum. *Southeast Asian Journal of Tropical Medicine and Public Health*, 1998, *29*, 579-583.

ITAGAKI T., SAKAMOTO T. & ITAGAKI H. Analysis of *Fasciola* sp. antigen by enzyme-linked immunotransfer blot using serum from experimentally and naturally infected cattle. *Journal of Veterinary Medical Science*, 1995, *57*, 511-513.

MALEEWONG W., WONGKHAM C., INTAPAN P.M. & PIPITGOOL V. *Fasciola gigantica* specific antigens: purification by a continuous-elution method and its evaluation for the diagnosis of human fascioliasis. *American Journal of Tropical Medicine and Hygiene*, 1999, *61*, 648-651.

MARTÍNEZ-MORENO A., MARTÍNEZ-MORENO F.J., ACOSTA I., GUTIÉRREZ P.N., BECERRA C. & HERNÁNDEZ S. Humoral and cellular immune responses to experimental *Fasciola hepatica* infections in goats. *Parasitology Research*, 1997, *83*, 680-686.

MBUH J.V. & FAGBEMI B.O. Antibody and circulating antigen profiles before and after chemotherapy in goats infected with *Fasciola gigantica*. *Veterinary Parasitology*, 1996, *66*, 171-179.

MOREAU E., CHAUVIN A. & BOULARD C. Lack of humoral and cellular responses against glutathione S-transferases in *Fasciola hepatica* experimentally infected sheep. *Veterinary Research*, 1998, *29*, 585-591.

OLDHAM G. Immune responses in rats and cattle to primary infections with *Fasciola hepatica*. *Research in Veterinary Science*, 1985, *39*, 357-363.

POITOU I., BAÉZA E. & BOULARD C. Kinetic responses of parasite-specific antibody isotypes, blood leucocyte pattern and lymphocyte subsets in rats during primary infestation with *Fasciola hepatica*. *Veterinary Parasitology*, 1993, *49*, 179-190.

ROBERTS J.A., ESTUNINGSIH E., WIEDOSARI E. & SPITHILL T.W. Acquisition of resistance against *Fasciola gigantica* by Indonesian thin tail sheep. *Veterinary Parasitology*, 1997, *73*, 215-224.

RODRIGUEZ-PÉREZ J. & HILLYER G.V. Detection of excretory-secretory circulating antigens in sheep infected with *Fasciola hepatica* and with *Schistosoma mansoni* and *F. hepatica*. *Veterinary Parasitology*, 1995, *56*, 57-66.

RUIZ-NAVARRETE M.A., ARRIAGA C., BAUTISTA C.R. & MORILLA A. *Fasciola hepatica*: characterization of somatic and excretory-secretory antigens of adult flukes recognized by infected sheep. *Revista-Latinoamericana-de-Microbiologia*, 1993, *35*, 301-307

SANTIAGO N. & HILLYER G.V. Antibody profiles by EITB and ELISA of cattle and sheep infected with *Fasciola hepatica*. *Journal of Parasitology*, 1988, *74*, 810-818.

SPITHILL T.W., SMOOKER P.M. & COPEMAN D.B. *Fasciola gigantica*: Epidemiology, control, immunology and molecular biology, *in*: Fasciolosis. Dalton J.P. (ed), CAB International, Wallingford, UK, 1999a, 465-525.

SPITHILL T.W., SMOOKER P.M., SEXTON J.L., BOZAS E., MORRISON C.A., CREANEY J. & PARSONS J.C. Development of vaccines against *Fasciola hepatica*, *in*: Fasciolosis. Dalton J.P. (ed), CAB International, Wallingford, UK, 1999b, 377-410.

Reçu le 20 juin 2003
Accepté le 21 janvier 2004