SHORT COMMUNICATION

AN ATTEMPT TO VACCINATE SHEEP WITH WHOLE HOMOGENATE OF THIRD-STAGE AND ADULT *HAEMONCHUS CONTORTUS.*

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SUMMARY: Six, 7 month old Scottish Blackface male sheep, were randomly divided in three groups of 2 animals each. Sheep in group A and B were vaccinated with 7.0mg of protein from whole larval (A) or adult (B) *H. contortus* homogenates, emulsified with Freund's complete adjuvant, and boosted five weeks later with 1.75mg of the respective homogenates in Freund's incomplet adjuvant. Sheep in Group C remained as non-vaccinated controls. The animals were all challenged five weeks after boost, orally, with 10.000 L3 and sacrificed 28 days later. One L3 vaccinated animal (A13) showed a 63% reduction in worm burden compared to the average of the controls and a correlation between reactivity to the surface of L3 and the lowered worm burden on immunofluorescence assay was also observed. No effect of immunization with adult homogenates was observed. The IgG titre measured by ELISA was not correlated with the level of worm burden after challenge. Western blots of the sera from Group A animals show that they recognize a series of polypeptides with a high molecular weight component being predominant.

KEY WORDS: Vaccination, *Haemonchus contortus*, sheep.

Haemonchus contortus is a major pathogenic gastrointestinal parasite of worldwide significance and economic importance. It has been shown that sheep which have been exposed to multiple infections of *H. contortus* become resistant to reinfection (CHRISTIE *et alii*, 1978). Resistance can also be stimulated by immunization with irradiated infective larvae of *H. contortus*, but only gives protection to lambs over 6 months of age (URQUHART *et alii*, 1966). Other attempts to stimulate protection have been made using a series of infections with small numbers of viable non-irradiated larvae, and it has been shown that lower worm burdens and faecal egg counts are achieved on challenge (DONALD *et alii*, 1969; DARGIE & ALLONBY, 1975; ADAMS & BEH, 1981). BARGER *et alii* (1985) found that six month old lambs develop a strong immunity to challenge between four and seven weeks after the beginning of a continuous infection. Immunity was also stimulated in young lambs after several massive infections, each truncated at the 4th stage by anthelmintic treatment (CHRISTIE & BRAMBELL, 1966).

Recently, trials have been made to induce protective immunity by vaccination with secreted/excreted products or somatic homogenates (ADAMS *et alii*, 1982; MUNN *et alii*, 1987; NEILSON & VAN DE WALLE, 1987; BOISVENUE *et alii*, 1987; ADAMS, 1989) and some evidence that protection can be induced by vaccination with these antigenic components has been obtained.

The purpose of this study was firstly to determine whether vaccination of sheep with L3 or adult homogenates of *H. contortus* was capable of inducing protection from challenge with 10,000 L3 and secondly to evaluate the immune response to the homogenates by determination of worm burdens and the level of protective serum antibodies.

Six, 7 month old Scottish Blackface male sheep (raised and maintained free from parasites) were randomly divided in three groups of 2 animals each. Sheep in Group A and B were vaccinated with 7 mg of protein from whole larval (A) or adult (B) homogenates, and boosted five weeks later with 1.75 mg of the respective homogenates. For immunization, L3 and adult homogenates were emulsified with Freund's complete (vaccination) and incomplete (booster) adjuvant in a 4:6 proportion (Freund's adjuvant:homogenates) and the emulsion was injected subcutaneously. Sheep in Group C remained as non-vaccinated controls. All animals were challenged orally, 5 weeks after boost, with 10,000 L3 and sacrificed 28 days after challenge for the determination of worm burdens. The sheep were weighed at the time of immunization, boost, challenge and slaughter. Faecal egg counts and PCV were monitored weekly during the immunization and prepatent periods and twice a week once infection was detected.

After necropsy, sixty male and sixty female parasites were recovered from each sheep, and their dimensions measured. The values from each group were analyzed by analysis of

variance and the means compared by the Newman Kleus multiple range test. Differences were considered significant at $p<0.05$.

Parasite antigens of third stage larvae and adult were obtained as described by KEITH *et alii* (1990).

Samples, standardized for protein content, were resuspended in sample buffer (0.85M Tris, 3% SDS, 10% glycerol, 0.01M EDTA pH=7) with 2-mercaptoethanol and 0.1% bromophenol blue, heated for 3 minutes at 100°C and electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), according to the method of Laemmli (1970). Eletrophoretic transfer of proteins to nitrocellulose membranes was performed as described by TOWBIN *et alii* (1979) at 60V for 3h. The membranes were blocked by 10% horse serum and 5% skimmed milk in Tris-saline (TBS, 10mM Tris, 0.9% NaCl pH=7.4) for 3h at room temperature. The blotted membranes were incubated with antisera (1:200 dilution) overnight at room temperature followed by incubation with 1:200 diluted anti-sheep IgG labelled with peroxidase (Sigma) for 120min. Finally, peroxidase substrate (20mg 4-cloro-1-napthol, 20ml methanol, 80ml TBS and 100µl hydrogen peroxidase) was added to the strips and upon completion of color development, the reaction was stopped by adding TBS.

ELISAs were used for the detection of specific antibodies against larval and adult antigens. The antigens used were the soluble extracts of L3 or adult *H. contortus* . The developing antibody was a anti-sheep IgG peroxidase-conjugate (whole molecule-Sigma). The microplates were coated with parasite antigen (5µg protein ml^{-1}) in 0.2M sodium carbonate-bicarbonate buffer pH=9.6, incubated overnight at 4°C and then blocked with 100µl of phosphate buffer saline (PBS) plus 4% skimmed milk per well, for 30 minutes at 37°C. Serum from weeks zero, 5 and 10 were diluted 1:50, 1:100, 1:500, 1:1000 and 1:2000 in PBS plus 2% skimmed milk, add to the plates, and incubated for the same time and at the same temperature as indicated above. Antibody-conjugate (100µl per well) diluted 1:1000 in PBS was added to the plates and incubated for 30 minutes at 37°C. Chromogen plus hydrogen peroxidase was used as a substrate and the reaction was terminated by addition of 100µl of 15% sulphuric acid per well. Between each step the plates were washed three times with PBS plus 0.05% Tween-20. The optical densities were measured in a ELISA reader (Titerlek Multiskan) at a wavelength of 492nm. The sera were analysed in duplicate. Four positive *H. contortus* sera (from sheep that received twice infective doses of the parasite) and four negative controls (pre-immunization serum) were included at 1:50 dilution in every plate. Means were determined for the absorbancy values obtained from the negative control and values greater than twice of this value were interpreted as positive and values falling within the range of the control were considered negative. The same procedure was adopted for the serum from weeks zero to 10 (immunization period), where only 1:50 dilution was used.

Adults or exsheated L3 were resuspended in 200µl of PBS in 1.5 ml eppendorf tubes. The serum to be tested and a pre-immunization serum sample (negative control), diluted 1:25, were added, incubated for 30 minutes on ice and then washed five times in PBS before incubation for 30 minutes with fluorescein conjugated anti-sheep IgG (FITC) diluted 1:25 in PBS. Unbound FITC-antibody was removed withthree washes in PBS and the parasites mount on slides and examined using a 580 FITC filter under ultraviolet light. The stage and species specificity of the antibodies were examined using sera from sheep that were immunized with L3 and adult homogenates.

Table 1 - Eggs per gram of faeces and abomasal worm counts after a challenge with 10.000 **H. contortus** L3 in sheep vaccinated with extracts of L3 or adult parasites.

GROUP	TREATMENT	EGGS PER GRAMS OF FAECES				WORM BURDENS
		DAYS AFTER INFECTION				
		16	21	24	28	
A	L3 EXTRACT					
13		0	100	1500	2700	1410
16		0	0	4200	20000	5870
B	ADULT EXTRACT					
14		0	0	200	6900	6400
17		50	3700	17400	32100	5520
C	CONTROL					
15		0	4200	30900	58200	4260
18		0	50	1000	1150	3230

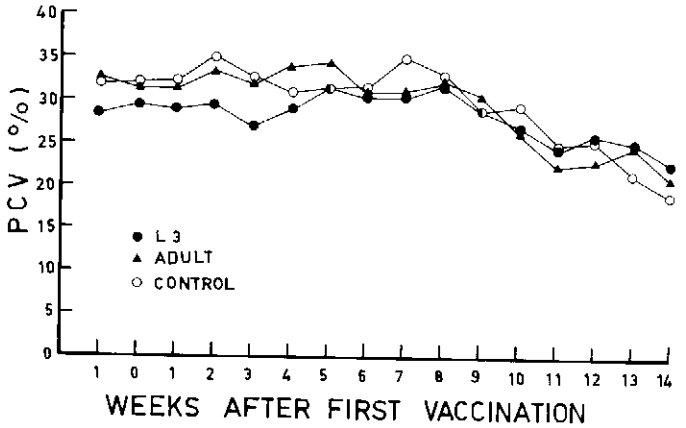

Fig. 1 - Mean weekly PCV (%) values of the sheep vaccinated with whole homogenate of L3 and adult of *Haemonchus contortus* and the respective controls .

RESULTS AND DISCUSSION

During the vaccination regimen and after challenge, all sheep gained weight steadily. The PCV's of all animals were normal during the immunization period but after challenge the values showed reductions that were correlated with the number of worms recovered at necropsy (Fig.1). The epg determination from days 16 to 28 after challenge and worm burdens present at necropsy are shown in Table 1. No protection was found based on worm burdens. One L3 vaccinated animal (A-13, Table 1) showed a 63% reduction in

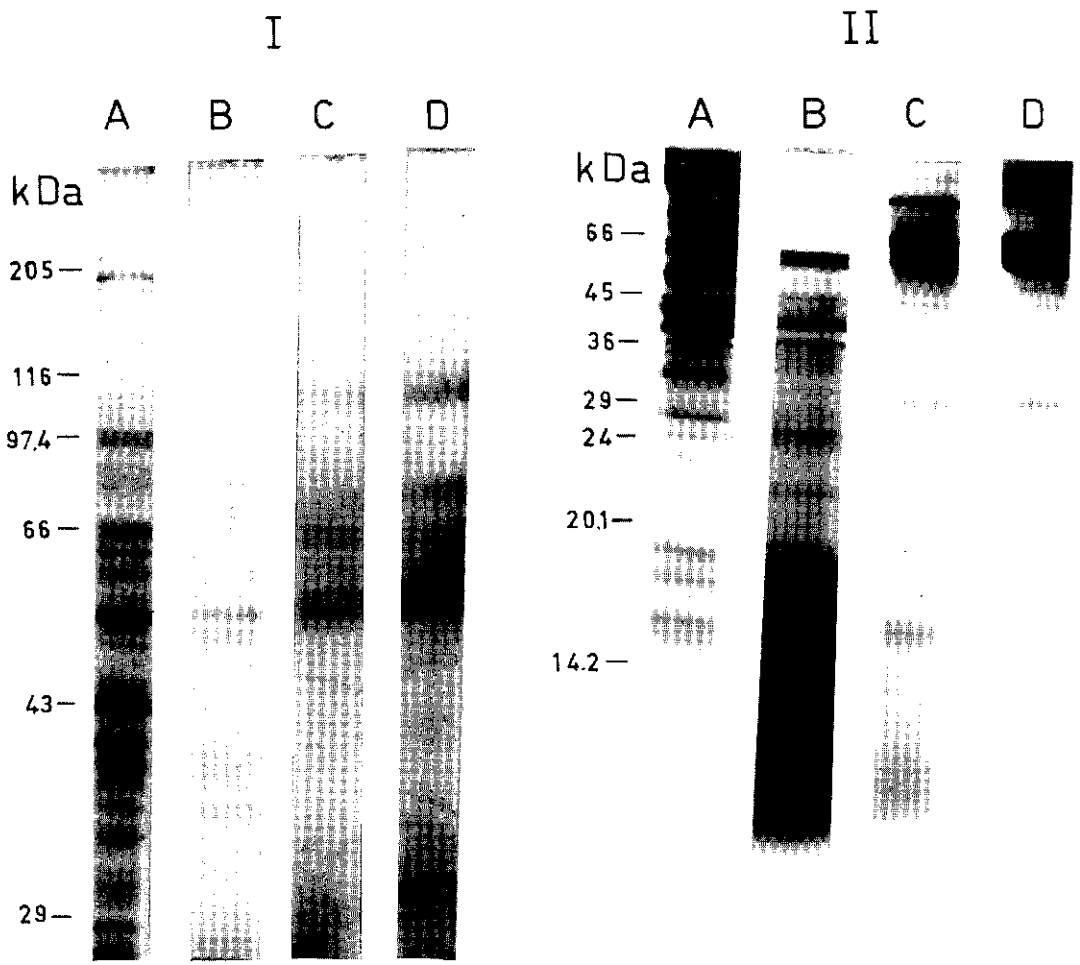

Fig. 2 (I) - 10% SDS-PAGE and (II) - 15% SDS-PAGE soluble protein pattern of *H. contortus* L3 (A), adult (B), adult pellet plus sodium dodecyl sulphate (C), and adult pellet plus 2-mercaptoethanol (D) stained with Coomassie Blue.

worm burden compared to the avarage of the controls, but the others, immunized with either L3 or adult extracts, showed higher worm burdens than the controls. Both male and female parasites from group A were statistically smaller than those isolated from the group B and control ($p<0.05$), with mean of 1.33±0.02, 1.39±0.01 and 1.42±0.03 for the males and 1.38±0.02, 1.86±0.03 and 1.98±0.03 for the females from groups A, B and C respectively. The antibody titres in the two immunized groups (ELISA) were high and the titration test showed that dilution of 1:50 appear to be most suitable for the present test. Using larval or adult soluble antigen extracts, the IgG antibodies against *H. contortus* were detected in the first week after immunization and the level of antibody continue to increase after boosting, remaining constant until challenge. In the control animals the level of antibody increased after challenge but was lower than that in the animals of group A and B. The IgG response, using larval or adult homogenate, showed a similar pattern with a peak after boosting. The antibody titre by ELISA was not correlated with the level of worm burden post challenge (data not shown). By using the same technique, NEILSON & VAN DE WALLE (1987) found enhanced serum antibody titres in vaccinated or primary infected and challenged lambs, but the titres were not related to resistance to challenge with *H. contortus*.

In order to define the antigens recognised by the sera from the three groups of animals, Western blots of L3 and adult homogenates were undertaken. Initially, SDS-PAGE gels were run and stained with Coomasie blue to define the proteins extracted in the soluble L3 homogenate fraction and the various fractions of the adult homogenate. The results are shown in Fig.2. Twenty peptides with molecular weights between 205 KDa and 29 KDa where detected when L3 antigens were used (Fig.2-I, track A.). Using soluble adult antigens, despite the higher protein concentration used (100µg) only 4 bands could be detected (Fig.2-I, track B) although a greater number of bands were detected when SDS or ME extracts were applied (Fig.2-I, tracks C and D). Using 15% PAGE gels a range of lower molecular weight polypeptides were detected in the L3 homogenates (Fig.2-II track A). The adult buffer soluble extracts show a number of polypeptides but perhaps more significantly an intense broad smear below 20 KDa (Fig.2-II, track B) which suggests that the adult buffer soluble extract may have been degraded by

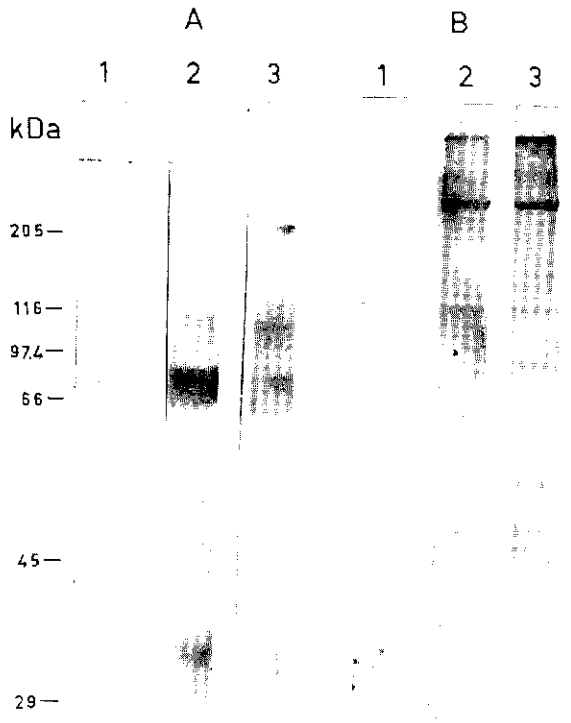

Fig. 3 - (A) Immunoblotting of adult *H.contortus* soluble proteins with pre-immune serum from animals immunized with adult homogenate (B14) (Lane 1), after boost (Lane 2) and after challenge (Lane 3). (B) Immunoblotting of soluble protein of L3 *H. contortus* recognized by sera of L3 homogenate immunized animals (A13): pre-immune (Lane 1), after boost (Lane 2), and after challenge (Lane 3).

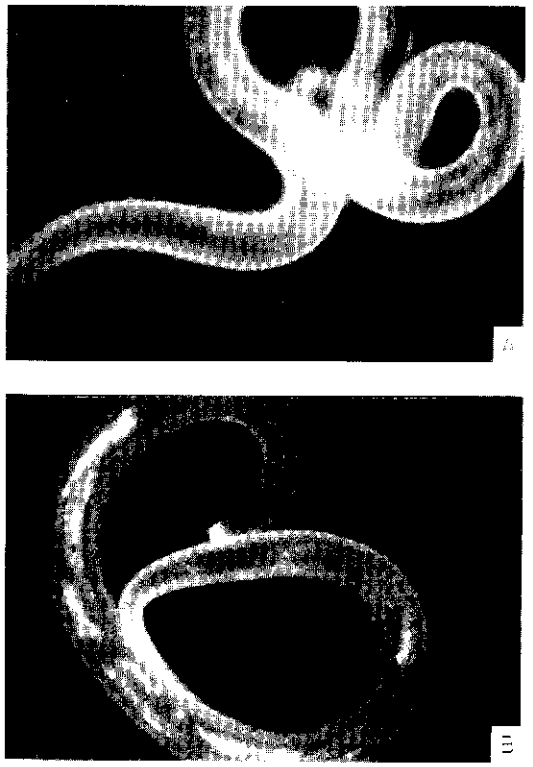

Fig. 4 - Fluorescence photomicrographs of exsheathed *H. contortus* L3 larvae treated with: (A) serum from animal 13 vaccinated with L3 homogenate and (B) serum from non-vaccinated control sheep (C15) followed by incubation with anti sheep fluorescein isothiocyanate conjugated antibody.

proteases not inhibited by the cocktail of inhibitors used, although this does not occur in the SDS and ME adult soluble extracts (Fig.2-II, tracks C and D).

On the basis of this analysis there are some common polypeptides between the L3 and adult homogenates, but overall the two extracts give very distinct patterns.

Western blots of both adult (Fig.3A) and SDS-ME adult homogenates (data not shown) as antigen showed only a few polypeptides recognised by antibody, however the intensity of the pattern increased after boost and challenge (Fig. 3A, tracks 2 and 3). These blots show that the immune sera recognise five main polypeptide species. Immunoblots of L3 homogenates and sera from L3 immunized animal (sheep 13) (Fig.3B), showed one main polypeptide >205 KDa. Recognition of this polypeptide was first detected two weeks after first immunization and the intensity of staining incresed after boost. Animal 13, group A, showed a greater intensity of staining than animal 16, for the same group, and the former animal showed the lowest worm burden after challenge.

On immunofluorescence, only the serum from animal 13 with the lowest worm burden, recognized the surface of the infective stage of *H. contortus* (Fig.4).

Variation between experimental animals influenced by certain host-parasites systems were already described and called responders and non-responders (WINDON & DINEEN, 1984 and ADAMS, 1989), and probably animal 13 has a characteristic of responder and showed a distinct behavior.

The results presented are a preliminary set of experiments aimed at defining the antigens recognized by sheep, immunized with extracts of adult and infective larval stages of *Haemonchus contortus* and investigating whether such responses play a role in protection. The results obtained on challenge show that there is no effect of immunization with adult homogenates and this is reflected by the lack of reactivity of the antibody from these animals with the surface of adults. The results on challenge after immunization with L3 homogenates are difficult to interpret as one animal shows a little effect relative to controls. The results of immunofluorescent assay using antibody from these sheep, show a correlation between reactivity to the surface of L3 and the lowered worm burden. Western blots of these sera show that they recognise a series of polypeptides with a high molecular weight component being predominant. The strength of signal to this polypeptide is correlated with the lowering of worm burden on challenge. Clearly further research is required to establish whether these findings are valid using larger numbers of animals and examining the effect of immunizing dose and adjuvant. NEILSON & VAN DE WALLE (1987) did a dose response experiment covering a dose range of 0.001mg to 10 mg protein from larvae homogenate and excretions - secretions products of *H. contortus*. They found maximum antibody

titres, measured by ELISA with the 1mg protein dose and this is another important aspect to be considerated in future trials with the use of different routes of inoculation and different adjuvants that could be more effective.

SUMÁRIO

Seis cordeiros machos, da raça Scottish Blackface com 7 meses de idade foram divididos em três grupos de 2 animais cada. Os cordeiros foram vacinados com 7,0mg de proteína de um homogenado constituído de larvas infectantes (grupo A) e adultos de *H. contortus* (grupo B), emulsificados com adjuvante completo de Freund e revacinados com 1,75mg dos respectivos homogenados em adjuvante incompleto de Freund. Os cordeiros do grupo C permaneceram como controles, não vacinados. Todos os animais foram desafiados 5 semanas mais tarde, com 10.000 L3, via oral e sacrificados 28 dias depois. Um animal vacinado com homogenado de L3 (A13) apresentou uma redução de 63% no número de vermes estabelecidos quando comparado à média dos controles e também observou-se uma correlação entre reatividade à superfície de L3 com o número de vermes estabelecidos quando os soros foram testados pela técnica de imunofluorescência. Os homogenados de adultos não apresentaram nenhum efeito imunizante. Os títulos de IgG medidos pela técnica de ELISA não apresentaram correlação com o número de vermes obtidos na necrópsia. Western blots dos soros dos animais do grupo A, mostraram que estes reconheceram uma série de polipeptídeos, com predominância daqueles com alto peso molecular.

PALAVRAS CHAVE: vacinação, *Haemonchus contortus*, ovinos.

ACKNOWLEDGEMENTS

We wish to acknowledge the technical assistance of the Department of Parasitology, Veterinary School University of Glasgow and to Prof. James L. Duncan for helpful criticism during the experiment. S. M. Gennari was supported by a fellowship from Brazilian Council for Scientific and Technological Development (CNPq).

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(Received, July 28, 1992)