Protective immunity to the blood-feeding nematode *Haemonchus contortus* induced by vaccination with parasite low molecular weight antigens

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SUMMARY

Partially purified low molecular weight antigens obtained by gel filtration of whole worm homogenates or total adult excretory–secretory (ES) products were tested in a vaccination experiment to determine their ability to induce protective immunity against *Haemonchus contortus* in sheep. Sheep were challenged with 20000 infective 3rd-stage larvae. One animal in the low molecular weight vaccinated group showed no protection against *H. contortus*, whereas the 4 other sheep in this group showed a mean reduction of 99·9% in faecal egg counts and of 97·6% in abomasal worm burden compared to the non-vaccinated controls and the adjuvant controls. The ES-vaccinated sheep showed a 32·2% reduction in parasite egg production and a 63·7% reduction in abomasal worm counts. Analysis of the humoral immune responses revealed no significant differences in antibody recognition of putative protective antigens between the protected and non-protected vaccinated animals. However, a marked lower lymphocyte proliferation response was found in non-protected sheep.

Key words: nematodes, *Haemonchus contortus*, low molecular weight antigens, sheep, vaccination.

INTRODUCTION

Infections with gastro-intestinal nematodes that feed on blood are a major constraint on sheep and goat production in many parts of the world. The control of gastro-intestinal parasitism has depended largely on pasture management and the use of anthelmintics. However, clean pastures are not readily available under intensive grazing conditions. Moreover, there is an increasing occurrence of parasites resistant to the action of anthelmintics (Düwel, 1987; Jackson, 1993; Waller, 1994). Thus, the need to develop alternative control methods, like vaccines, is apparent. In this respect, the identification of specific antigens that can induce protective immune responses to these parasites is crucial.

For the blood-sucking trichostrongylid *Haemonchus contortus* use of partially purified gut antigens as immunogen afforded a degree of protection against this parasite to both goats and sheep (Jasmer & McGuire, 1991; Tavernor *et al*. 1992*a*, *b*; Munn *et al*. 1993*a*, *b*; Smith & Smith, 1993; Smith *et al*. 1993; Jasmer *et al*. 1993). The protection induced by this type of vaccination is artificial and is probably quite different from naturally acquired immunity (Smith, 1993). The latter type of immunity is acquired after a continuous or seasonal exposure to, or after moderate experimental trickle infections with, infective larvae (Smith, 1977; Barger *et al*. 1985; Schallig, van Leeuwen & Hendrikx, 1995*b*). It has been demonstrated that a protective natural immune response is associated with the humoral recognition of low molecular weight *H. contortus* antigens. In particular, a 15 and a 24 kDa antigen in adult somatic extracts as well as in adult excretory–secretory (ES) products are specifically recognized by sera of *H. contortus*-immune sheep (Schallig, van Leeuwen & Hendrikx, 1994; Schallig *et al*. 1995*b*). In the present study it was tested whether vaccination with low molecular weight antigens can induce protection against *H. contortus*. However, a problem is that the 15 and 24 kDa antigens are not readily available as immunogen. In order to obtain sufficient amounts of low molecular weight antigens for vaccination, the 15 and 24 kDa antigens had to be (partially) purified by gel filtration from adult somatic extracts. It was, at present, not possible to purify the 15 and 24 kDa from the less complex *H. contortus* ES material. This is due to the fact that *in vitro* maintenance of adult *H. contortus* usually yields limited amounts of ES products. We did, however, also test total ES material as immunogen, because it has been demonstrated that this material in its own right can provoke strong humoral and cellular immune responses in sheep (Lightowlers & Rickard, 1988; Schallig *et al*. 1995*b*).

MATERIALS AND METHODS

Parasite and preparation of parasite antigens

A benzimidazole-sensitive strain of *H. contortus*, originally obtained from the Moredun Research

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Institute (Edinburgh, UK) was used. Adult *H. contortus* were harvested from the abomasum after sacrifice of donor sheep which had received a controlled infection with 20000 infective 3rd-stage larvae (L3). The collected worms were subsequently washed 5 times in phosphate-buffered saline (PBS, pH 7·4), containing penicillin (500 I.U./ml) and streptomycin (5 mg/ml) and either stored frozen at −80 °C until further use or maintained *in vitro* for the production of ES material (Schallig *et al*. 1994).

Water-soluble proteins were isolated from adult worms as previously described (Schallig *et al*. 1995*b*).

In order to obtain sufficient amounts of low molecular weight antigens for vaccination, the 15 and 24 kDa antigens were partially purified from adult somatic extracts. Molecular weight separation was achieved by gel filtration on a HI Load 26/60 Superdex 75 preparative grade column (Pharmacia Biotech, Roosendaal, The Netherlands) equilibrated with PBS. A 2 ml sample containing approximately 25 mg protein was loaded on the column and proteins were eluted at a flow rate of 1 ml/min and 2 ml fractions were collected. A low molecular weight gel filtration calibration kit covering the molecular weight range of 13·7 to 67 kDa (Pharmacia Biotech) was used to calibrate the column. The purification process was monitored by SDS–PAGE and Western blotting using pooled serum from *H. contortus* hyperimmunized sheep (Schallig *et al*. 1995*b*). The purification procedure yielded a 15 and 24 kDa antigen enriched fraction (EF15/24) that was used for vaccination and as test antigen in the immunological assays.

Protein concentrations were determinated using the bicinchonic assay (Pierce, Rockford, USA). Samples were stored at −20 °C.

Immunization trial and parasitology

Texel sheep, 8 months of age, reared and housed indoors in conditions to exclude accidental infections with helminths (Schallig *et al*. 1995*b*) were used. Five groups each consisting of 5 animals were made. Group 1 served as a non-infected control group. Group 2 received only a challenge infection. Group 3 was used as an adjuvant control (2 ml of PBS containing 5 mg dimethyl dioctadecyl ammonium bromide, DDA) and received also a challenge infection. Group 4 was vaccinated 3 times with 100 μg ES antigens dissolved in 2 ml of DDA adjuvant and was subsequently challenged. Finally, group 5 was vaccinated 3 times with 100 μg EF15/24 dissolved in DDA adjuvant and was also challenged. All infections were administered s.c. at week 1, 4 and 5 of the experiment. The challenge infection of 20000 L3 was given orally at week 6.

Faecal samples were collected at week 0 and weekly from week 6 to the end of the experiment (week 10). Faecal egg counts were made using the McMaster technique and expressed as eggs per gram faeces (epg).

At the end of the trial all sheep were slaughtered and the abomasum was immediately removed, opened and the contents collected in a container. The empty abomasum was washed thoroughly with 0·9 % saline solution to remove adhering worms. The abomasum was next soaked in 0·9 % saline for 4 h at 37 °C. After soaking, the abomasum was washed thoroughly and the washings were sampled. Worm counts were made on 1/50 aliquots of both washings and abomasal content according to previously described methods (Eysker & Kooyman, 1993). Counted worms were classified by their stage of development as indicated in Table 2. The data on abomasal worm counts and the faecal egg counts were statistically analysed using Student's *t*-test. Differences between the groups were considered significant at $P < 0{\cdot}05$.

Sodium dodecylsulphate–polyacrylamide gels (SDS–PAGE) and Western blotting

SDS–PAGE was done under non-reducing conditions using a 4 % stacking gel and 12·5 % running gel essentially as described (Schallig *et al*. 1994). Low molecular weight protein standards (Pharmacia LKB Biotechnology) were electrophoresed simultaneously. After electrophoresis, Western blots were done as described previously (Schallig *et al*. 1994), using horseradish peroxidase (HRP)-conjugated rabbit anti-sheep immunoglobulins (Dako, Copenhagen, Denmark) diluted 1:1500 as the detection system. Bound antibodies were visualized by adding 0·05 % 3,3 diaminobenzidine tetrachloride in PBS containing 0·01 % H_2O_2 (v/v). Sera obtained at week 10 of the experiment were used to probe the blots.

Enzyme-linked immunosorbant assay (ELISA)

Serum samples were collected weekly (from week 0 to 10) and tested in ELISA as described previously for *H. contortus* ES products (Schallig, Hornok & Cornelissen, 1995*a*). In brief, plates were coated overnight at 4 °C with antigen (ES or EF15/24) at a concentration of 2 μg/ml dissolved in a 0·06 M carbonate buffer, pH 9·6. Sheep sera were applied to the plates in a 1:200 dilution. Rabbit anti-sheep IgG1 (diluted 1:500) was used as secondary antibody and HRP-conjugated swine anti-rabbit immunoglobulins (1:1500 diluted) was used as conjugate. Finally, 100 μl of 1 mM 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) in 100 mM citrate/200 mM phosphate buffer (pH 5·0) with 0·01 % H_2O_2, was used to develop the assay. The optical density was measured at 405 nm using a Microplate reader Model 3550 (Bio-Rad, Richmond, USA).

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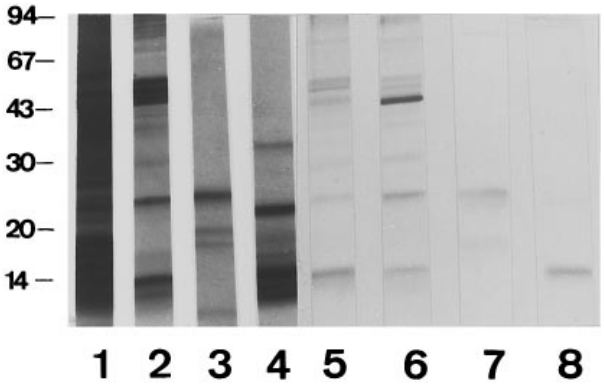

Fig. 1. Result of SDS–PAGE and immunoblotting using serum of *Haemonchus contortus* hyperimmunized sheep to detect the gel filtration fractions containing the 24 kDa or 15 kDa antigen: protein profile of total crude worm homogenate (1), of total ES material (2), of gel filtration fraction 36–38 (3), or of gel filtration fraction 52–54 (4), immunoblot of total crude worm antigens (5), of total ES material (6), of gel filtration fraction 37 (7), or of gel filtration fraction 53 (8). The molecular weights of standards are indicated in kDa.

Lymphocyte proliferation assay (LPA)

Lymphocytes were collected at weeks 0, 6, 8 and 10 in order to perform LPA. Cells were obtained from sheep by bleeding them from the jugular vein into vacutainers (Becton Dickinson, Meylan Cedex, France) containing 143 units sodium heparin. Heparin blood was diluted 1:1 with PBS/heparin (10 I.E./ml). A mononuclear cell-enriched preparation was obtained and tested in LPA as described previously (Schallig *et al*. 1994). Parasite antigens (ES or EF15/24) were used at a concentration of 1 μg/ml in 100 μl volumes in triplicate. Concanavalin A at a final concentration of 10 μg/ml was added as a positive control for the test. To determine the basic level of proliferation, 100 μl of complete medium was used. The cells were cultured for a total of 4 days including a final 18 h pulse with 0·5 μCi [methyl]^{3}H-thymidine (Amersham, Den Bosch, The Netherlands) per well. Cells were harvested, unincorporated label was removed by a wash step and activity counted in the presence of scintillant in a LKB 1205 Betaplate scintillation counter. The results of the LPA are presented as stimulation indices (SI):

$$\text{SI} = \frac{\text{cpm (experimental)}}{\text{cpm (RPMI control)}}$$

Statistical significance was determined by Student's *t*-test.

RESULTS

Purification of parasite low molecular weight antigens

SDS–PAGE combined with Western blotting revealed that the 24 kDa and the 15 kDa antigens were present in fractions 36–38 and fractions 52–54, respectively (Fig. 1) with some contamination of other proteins. These fractions were pooled (= EF15/24) and used for vaccination and in the immunological tests.

Immunization trial and parasitology

The results of the faecal egg counts at week 10 are presented in Table 1. The epg of the non-infected control group 1 remained zero throughout the experiment. The ES-vaccinated group 4 showed a 32·2 % reduction in faecal egg counts when compared to those of group 2. However, the mean epgs of groups 2, 3 and 4 did not differ significantly ($P > 0{\cdot}05$) from each other. With the exception of 1 animal (sheep 470), all sheep in the EF15/24-vaccinated group 5 had lower faecal egg counts than the sheep in groups 2 and 3. If the epg data from sheep 470 are omitted, the mean epg of this group is significantly reduced ($P < 0{\cdot}05$) when compared to those of groups 2, 3 or 4. However, if the faecal egg counts of sheep 470 are included in statistical analysis, the mean reduction of group 5 is 21·1 % which is not statistically significant.

No worms were found in the non-infected control group 1; therefore this group is left out in Table 2. The infected control groups 2 and 3 did not show significantly different numbers of abomasal worm counts ($P > 0{\cdot}05$). Numerically lower worm counts were observed in the ES-vaccinated group 4 when compared with groups 2 and 3. However, the mean differences between groups 2, 3 and 4 were not statistically significant ($P > 0{\cdot}05$). As expected on the basis of the epg data, the EF15/24-vaccinated group 5, with the exception of sheep 470, showed a reduction of both male and female abomasal worm counts compared with the other groups. When the data of sheep 470 were omitted from statistical analysis, this reduction (97·6 %) in worm burden is significant ($P < 0{\cdot}05$). On the other hand, if the abomasal worm count of sheep 470 is included in the statistical analysis, the mean reduction in worm burden of group 5 is 68·8 % which is not significant.

Analysis of the humoral immune response

The humoral responses of the sheep were monitored by ELISA and Western blotting. The time-course of the mean IgG1 serum antibody responses of the 5 groups tested against ES antigens or EF15/24 antigens are shown in Fig. 2A and B, respectively. Whatever antigen was tested, the antibody levels in the non-infected control group 1 were never elevated during the entire experiment. A constant increase in antibody level against ES antigens was observed in the ES-vaccinated group 4 from 2 weeks after the first injection and each subsequent vaccine injection boosted the humoral immune response (Fig. 2A). A

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Table 1. Results (individual data and group means ± S.E.) of faecal egg counts at week 10

(The reduction of egg counts is expressed as % of the mean number of eggs found in group 2.)

	Group 1	Group 2	Group 3	Group 4	Group 5
	0	3080	3580	40	0
	0	5360	3770	4370	20
	0	3250	4520	5450	13530*
	0	2890	1020	1780	0
	0	2600	3450	0	0
Mean	0[a]	3436[b]	3268[b]	2328[b]	5[a]
S.E.	0	493	592	1115	5
Reduction	N.A.	N.A.	4·9 %	32·2 %	99·9 %

* Data are omitted from statistical analysis (sheep 470).
[a, b] Means sharing a common letter do not differ significantly ($P < 0{\cdot}05$).
N.A., Not applicable.

Table 2. Results (individual data and group means ± S.E.) of abomasal worm counts (EL4 = early L4 larvae; L4/L5 total number of L4 and L5 larvae) at week 10

(No worms were found in the non-infected control group 1. The reduction is expressed as % of the mean number of worms found in group 2.)

	Group 2				Group 3			
	EL 4	L4/L5	Adult	Total	EL 4	L4/L5	Adult	Total
	0	50	2300	2350	0	300	4450	4750
	0	750	5500	6250	0	300	3300	3600
	0	150	3450	3600	0	150	3050	3200
	0	0	2100	2100	0	350	3050	3400
	50	400	950	1400	0	300	3700	4000
Mean	10	270	2860	3140[a]	0	280	3510	3790[a]
S.E.	10	138	770	855	0	34	263	274
Reduction				N.A.				< 0 %
	Group 4				Group 5			
	EL 4	L4/L5	Adult	Total	EL 4	L4/L5	Adult	Total
	0	0	200	200	150	0	0	150
	100	100	1500	1700	0	0	0	0
	0	0	1500	1500	0	300	4300	4600*
	0	50	2050	2100	0	0	0	0
	150	0	50	200	150	0	0	150
Mean	50	30	1060	1140[a]	75	0	0	75[b]
S.E.	32	20	396	396	44	0	0	44
Reduction	—	—	—	63·7 %	—	—	—	97·6 %

* Data are omitted from statistical analysis (sheep 470).
[a, b] Means sharing a common letter do not differ significantly ($P < 0{\cdot}05$).
N.A., Not applicable.

gradual increase in ES-specific antibodies was detected in group 5 which started after the third injection. The levels of antibodies directed against ES antigens in groups 2 and 3 increased after the challenge infection but remained lower than those found in the vaccinated animals.

Vaccination of group 5 with EF15/24 resulted in the emergence of specific antibodies after the second injection (Fig. 2B). There was a continuous increase in antibody level up to week 8. The antibody level against EF15/24 of sheep 470 was not significantly different from those of the other sheep in group 5. The antibody level against EF15/24 antigens in the ES-vaccinated group 4 started to rise slowly after the second immunization, reached its maximum level 1 week after challenge infection. Antibody levels against EF15/24 antigens did not increase in the infected control groups 2 and 3.

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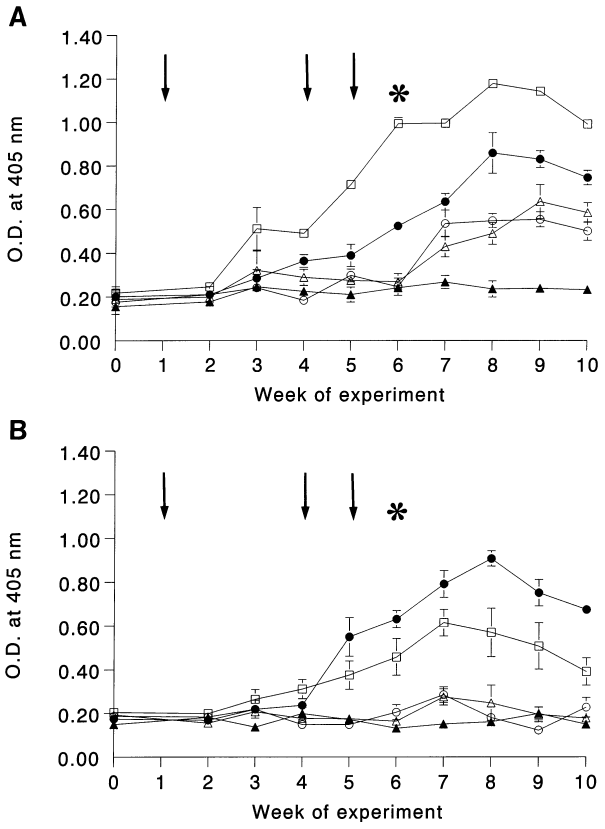

Fig. 2. (A) Mean (±S.E.) serum IgG1 antibody responses to *Haemonchus contortus* ES antigens of control group 1 (—▲—), 2(—△—) and 3 (—○—), ES-vaccinated group 4 (—□—) and EF15/24-vaccinated group 5 (—●—). (B) Mean (±S.E.) serum IgG1 antibody responses to *H. contortus* EF15/24 antigens of control group 1 (—▲—), 2(—△—) and 3 (—○—), ES-vaccinated group 4 (—□—) and EF15/24-vaccinated group 5 (—●—). Arrows indicate the days of vaccination and the asterisk the day of challenge.

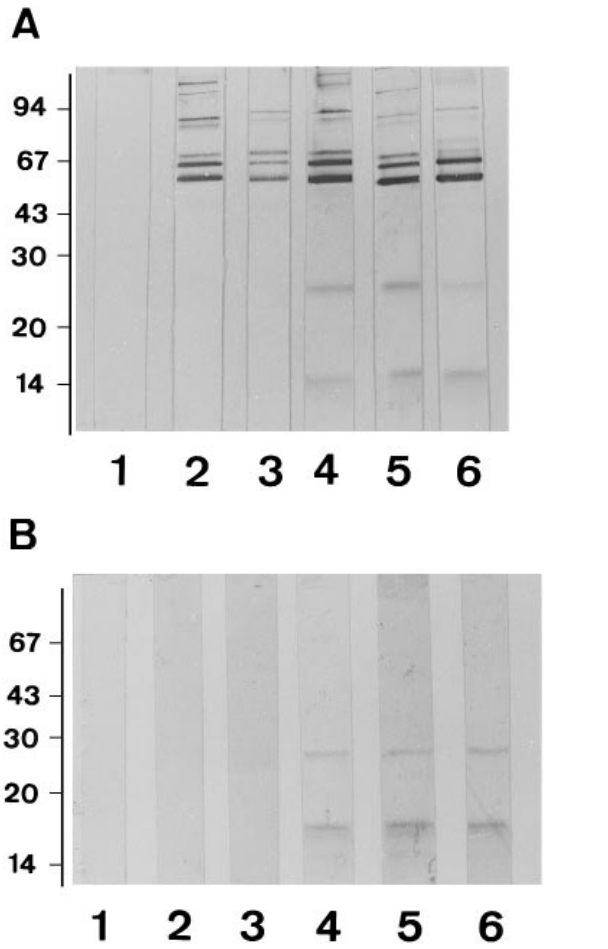

Fig. 3. (A) Western blot of *Haemonchus contortus* ES antigens probed with sera obtained from control groups 1–3 (lanes 1–3) and the vaccinated groups 4 and 5 (lanes 4 and 5) and of sheep 470 (lane 6) at the end of the experiment. A representative individual sample of each group is shown. (B) Western blot of *H. contortus* EF15/24 antigens probed with sera obtained from control groups 1–3 (lanes 1–3) and the vaccinated groups 4 and 5 (lanes 4 and 5) and of sheep 470 (lane 6) at the end of the experiment. A representative individual sample of each group is shown. The molecular weights of standards are indicated in kDa.

Western blot analysis revealed that sera from group 1 animals did not react with any of the antigens tested (Fig. 3A and B, lane 1). Sera obtained from groups 2 and 3 reacted with ES antigens with $M_r > 30$ kDa, but not with the lower molecular weight antigens (Fig. 3A, lanes 2 and 3). Sera obtained from groups 4 and 5, including sheep 470, reacted with the higher M_r antigens and with the 24 and 15 kDa ES products (Fig. 3A, lanes 4–6). Group 2 and 3 animals did not show a reaction with the EF15/24 antigens (Fig. 3B, lanes 2 and 3). These antigens were recognized by all sera obtained from groups 4 and 5, including sheep 470 (Fig. 3B, lanes 4–6).

Analysis of the cellular immune response

The results of the cellular responses, as measured in lymphocyte proliferation assays, are presented in Fig. 4A and B. The cells obtained from the non-infected control animals did not show proliferation responses to the two types of antigen preparation tested throughout the experimental period. The lymphocytes of the infected control animals (groups 2 and 3) showed slightly increased proliferation responses against the ES antigens at 2 and 4 weeks post-infection (i.e. weeks 8 and 10 of the experiment) and not before. A response to the EF15/24 antigens was not recorded in groups 2 or 3. Lymphocytes of ES-vaccinated sheep (group 4) showed significantly ($P < 0{\cdot}05$) increased responses to ES antigens from week 8. Their response against EF15/24 antigens was only significantly ($P < 0{\cdot}05$) increased at week 10. The EF15/24-vaccinated sheep (group 5) showed significantly ($P < 0{\cdot}05$) increased proliferative responses against both antigen preparations from week 8 and onwards. The mean proliferation response against EF15/24 antigens of the lymphocytes of group 5 was significantly greater than the response

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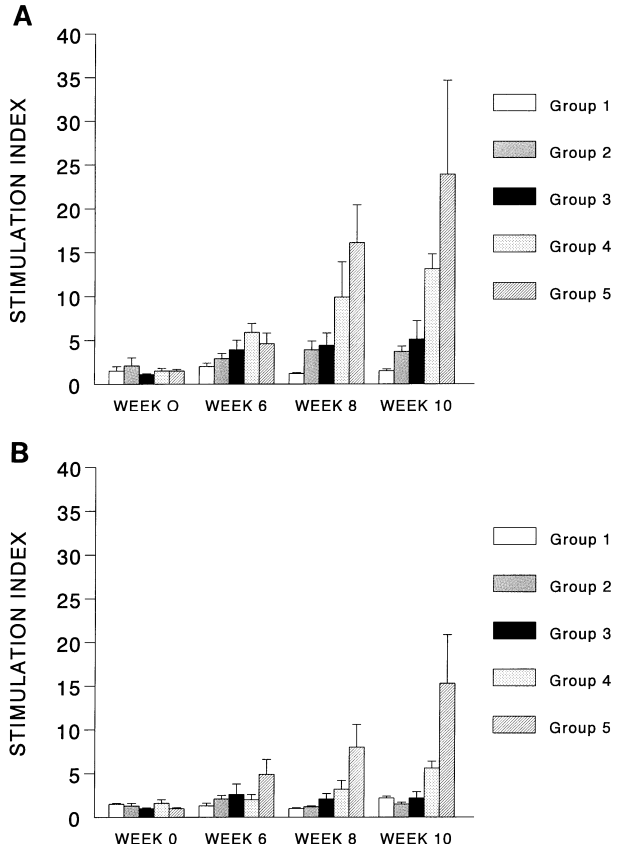

Fig. 4. (A) Cellular responses of sheep analysed by lymphocyte proliferation in the presence of *Haemonchus contortus* ES antigens. (B) Cellular response of sheep analysed by lymphocyte proliferation in the presence of *H. contortus* EF15/24 antigens. Values correspond to the mean stimulation indices ± S.E.

recorded in the ES-vaccinated group 4 ($P < 0{\cdot}05$). It is of interest to note that sheep 470 showed a marked lower proliferative response against the test antigens (SI of 3·4 against ES antigens and of 3·1 against EF15/24 antigens, respectively) compared to those of the other sheep in group 5. The SI of the other sheep ranged from 11·0 to 65·2 against ES antigens and from 4·7 to 32·9 against EF15/24 antigens.

DISCUSSION

The present study demonstrated that vaccination with lower molecular weight *H. contortus* antigens can induce a protective immune response against this parasite which is reflected in significant reductions in faecal egg counts and worm numbers. Both female and male worms were affected to the same extent and hardly any larval stages were found.

Within each group there was variation in the responses of individual sheep to challenge infection and, in the EF15/24-vaccinated group, in their immune response to injected antigen. This has been observed in other studies (e.g. Munn *et al.* 1993*b*; Tavernor *et al.* 1992*a*, *b*) and is most likely caused by the genetically determined variation in the ability of individuals to respond to parasites (Dineen, Gregg & Lascelles, 1978; Gray, 1987).

Serum transfer experiments have implicated antibody as the likely mechanism for gut antigen-induced immunity to *H. contortus* (Smith, 1993). This seems not to be the sole factor in the present study. Although antibodies obtained from ES-vaccinated animals recognized EF15/24 antigens on Western blots and in ELISA experiments, the sheep were protected to a lesser extent against *Haemonchus* than group 5 animals. In addition, antibodies from the non-protected sheep 470 in the EF15/24-vaccinated group also recognized these antigens. In contrast, *H. contortus*-immune sheep showed high lymphocyte responses to the low molecular weight antigens from this parasite. This suggests that the cellular part of the immune response also plays a role in protection against *H. contortus* as observed in the present experiment. This is supported by 2 other studies which demonstrated that lymphocytes from *H. contortus*-immune sheep showed strong proliferative responses especially against low molecular weight antigens (Haig *et al.* 1989; Torgerson & Lloyd, 1993).

An important question which has not been addressed in the present study is whether young lambs (< 6 months of age) can be protected with the low molecular weight vaccine. It is well known that young lambs are very sensitive to *H. contortus* infections, because they cannot mount protective responses (Urquhart *et al.* 1966; Neilson, 1975). Vaccination with the gut antigen induced also protection against *Haemonchus* in younger lambs (Tavernor *et al.* 1992*b*). It remains to be established whether the low molecular weight antigens also can induce protection against the parasite in younger animals.

The antigen preparation EF15/24 used to vaccinate the sheep contained not only the 15 kDa and 24 kDa antigens but also some other proteins. Therefore, it cannot be completely ruled out that these other proteins also contributed to the immunity against *Haemonchus*. In order to determine whether the 15 and 24 kDa alone are capable of inducing protection, vaccination with pure proteins must be carried out. However, it is difficult to obtain sufficient amounts of ES material from which the proteins of interest can be purified. Therefore, we will scale up the purification and use recombinant DNA technology to isolate and express the genes encoding these antigens and test the recombinant products in subsequent vaccination experiments.

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