

Protection in lambs vaccinated with *Haemonchus contortus* antigens is age related, and correlates with IgE rather than IgG1 antibody

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SUMMARY

Protection by vaccination with excretory–secretory products (ES) from *Haemonchus contortus*, containing predominantly proteins of 15 and 24 kDa, against an experimental challenge infection depends on the age of the sheep. Vaccinated sheep 9, 6 or 3 months of age were protected for 83%, 77% and –34%, respectively. There was a significant difference in ES-specific serum IgE response but not in IgG1 response, after the last vaccination between the different age groups. In the protected 9-month-old animals, there was an increase up to 18 times the pre-vaccination levels, while the increase in the unprotected 3-month-old animals was at most 1.4 times. The 6-month-old animals showed an intermediate increase of approximately six times the prevaccination level. There was no correlation within the 9-month-old sheep between ES-specific IgE levels and protection, measured as worm burden. However, when the different age groups were combined, there was a positive correlation ($r = 0.38$) between protection and ES-specific IgE levels 1 week after the vaccination. At the end of the experiment, peripheral blood eosinophils and mast cell counts in abomasal tissue were also significantly higher in the vaccinated and challenged 9-month-old sheep than in the vaccinated and challenged 3-month-old or than in the 9-month-old sheep with challenge, but without vaccination. Increased serum IgE levels, eosinophilia and mucosal mast cell hyperplasia are the hallmarks of a Th2 response and were all demonstrated in protected, older sheep, but not in unprotected, younger sheep.

Keywords ovine, IgE, nematode, *Haemonchus contortus*, ES, vaccine, immune response

INTRODUCTION

Haemonchus contortus is the most important parasite in sheep and goats worldwide. Anthelmintic resistance in this species is widespread. Recently strains of *H. contortus* have been found with resistance against all five groups of modern anthelmintics (van Wijk *et al.* 1997). Vaccination will become a useful tool in combating gastrointestinal nematode infections in ruminants. Vaccines against *H. contortus* which give partial protection are already under development. Hidden antigens, such as glycoprotein H11, have been employed in these vaccines, although they have a disadvantage in that there is no natural booster effect. However, they do protect young lambs against *H. contortus* (Tavernor *et al.* 1992). Vaccines based on 15/24 kDa proteins from excretory–secretory (ES) products do protect adult sheep (Schallig & van Leeuwen 1997b; Schallig *et al.* 1997a), but fail in sheep aged less than 6 months as demonstrated in this study. Protection of young sheep by a vaccine is important because this group is the most vulnerable to high infections in the field.

Hyporesponsiveness to helminth infections has been known in lambs for many years (Urquhart *et al.* 1966) and young mammals in general, although the immunological mechanism is not well understood. Lower numbers of CD4⁺ and CD8⁺ cells in blood and skin and lower levels of specific antibodies have been described in young lambs (Watson *et al.* 1994). Induction of IgE is regarded as an important factor in the host response to helminth infections in mammals (Jarrett & Miller 1982), but it was not until recently that monoclonal antibody against ovine IgE became available (Shaw *et al.* 1996, Kooyman *et al.* 1997). With the use of these antibodies, it was shown that there was an increase in the serum of parasite-specific as well as total IgE during nematode infection (Kooyman *et al.* 1997, Huntley *et al.* 1998, Shaw *et al.* 1998). In this study, we extended these studies with an emphasis on IgE responses

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and protection in sheep vaccinated against nematodes. The serum levels of IgE and IgG1 of 3, 6 and 9-month-old sheep are described after vaccination with ES proteins. Furthermore, the number of mast cells, eosinophils and cells positive for sheep mast cell protease (SMCP⁺) in the mucosa are determined and a correlation between protection, age and these parameters was made.

MATERIALS AND METHODS

Parasites and vaccine preparation

A benzamidazole-sensitive strain of *H. contortus* originally obtained from Moredun Research Institute (Edinburgh, UK) was used for isolation of ES proteins and for challenge infection. This strain has lost the ability for arrested development.

Preparation of the vaccine was as described previously (Schallig *et al.* 1997a). Briefly, adult *H. contortus* were kept for 24 h in RPMI 1640 and the ES products were collected from this medium. Isolation of the 15 and 24 kDa proteins was performed using a MONO Q column (Amersham Pharmacia, Uppsala, Sweden) and purification was monitored by standard SDS-PAGE analysis.

Immunization trial and parasitology

This paper is a sequel to a previous publication (Schallig *et al.* 1997a) and part of this experiment was described in this earlier work.

Texel lambs were weaned at approximately 3 months. Three different treatment groups were formed. Group V were animals vaccinated subcutaneously on week 3, 6 and 8 of the experiment with 50–100 µg 15/24 kDa obtained from ES of *H. contortus* formulated in 2 ml dimethyl dodecyl ammonium bromide (DDA), 2.5 mg ml⁻¹. This group was divided into three subgroups depending on age at the start of the experiment: 3 months (V3; *n* = 4), 6 months (V6; *n* = 3) and 9 months (V9; *n* = 9). Group C was the nonvaccinated group and was likewise divided in three identical age groups: (C3; *n* = 3) (C6; *n* = 3) and (C9; *n* = 5). Both group V and C were challenged at week 10. The 3-month-old lambs were challenged with 10 000 L3 and the 6 and 9-month-old sheep were challenged with 20 000 L3 of *H. contortus*. Finally, a negative control group (N) without vaccinations or challenge infection was formed. This group comprised only 9-month-old sheep (*n* = 5).

All sheep were killed at week 15 in a commercial slaughterhouse. The abomasum was opened immediately, the contents were collected and biopsies were taken (see below). Worm counts were performed according to Eysker & Kooyman (1993) on 1 : 50 aliquots.

Enzyme-linked immunosorbant assay (ELISA)

Serum samples were collected weekly and stored at -20°C until the assay was performed.

IgE

Total and specific IgE ELISAs were performed using a monoclonal antish sheep-IgE as described previously (Kooyman *et al.* 1997). Briefly, coating for ES-specific IgE was carried out overnight at room temperature with ES concentration of 5 µg ml⁻¹ in 0.06 M sodium carbonate (pH 9.6). Collection and preparation of ES antigen was as described previously (Schallig *et al.* 1994), modified with 10% TCA for precipitation of ES in the medium and 8 M urea for dissolving the ES pellet to concentrate and desalt the isolate. To determine total IgE, the plates were coated with polyclonal rabbit-anti-IgE. One ml of rabbit-anti-IgE serum was precipitated with 35% saturated ammonium sulphate (SAS) and the pellet was dissolved in 100 ml of coating buffer. The sheep sera were precipitated with SAS, the fraction precipitated between 30 and 50% SAS was dissolved in 1 volume dilution buffer (phosphate buffered saline with 0.1% gelatine and 0.05% Tween 20) and they were heated for 30 min at 56°C. These sera were applied to the plates without further dilution and incubated overnight at room temperature, followed by 1 h at 37°C. On each plate, a positive control serum was included. Subsequently, plates were incubated with monoclonal antish sheep-IgE (IE7) diluted 1 : 10, rabbit-antimouse HPO conjugate (DAKO, Denmark) diluted 1 : 1500 and ABTS/H₂O₂. Plates were read at 405 nm after 2 h.

IgG1

These ELISAs were performed as described previously (Schallig *et al.* 1997a). Coating with ES followed that described for IgE. The sample sera were diluted 1 : 100 and incubated for 1 h at room temperature. Rabbit-antisheep IgG1 (Goudswaard *et al.* 1980) was diluted 1 : 500 and swine-antirabbit HPO (DAKO, Denmark) was diluted 1 : 1500. ABTS/H₂O₂ was used as substrate and plates were read at 405 nm after 30 min.

Western blots

Western blots were made with 5 µg ES protein per lane as described previously (Kooyman *et al.* 1997). Dilution of the sera and antisera were the same as for the ELISA (see above) except for the sample sera, pooled or individual for IgE detection, which were diluted 1 : 6 for the blots. DAB/H₂O₂ was used as substrate.

Cell counts

Peripheral blood eosinophils were counted and histochemical methods were used to differentiate between the different cell types as described previously (Huntley *et al.* 1995). Mast cells were identified and counted after staining with Toluidine blue in 0.5M HCl. SMCP⁺ cells were identified and counted on basis of the reaction with affinity purified rabbit-anti-SMCP. SMCP is restricted to globule leucocytes and mast cells, and thus the numbers of SMCP⁺ cells are the combined numbers of these two cell types. Staining with carbol chromatrope made it possible to differentiate and count the eosinophils. The counts were performed on three separated sections of the same fold. The results were expressed as cell numbers per 0.2 mm².

Statistical analysis

Statistical analysis was carried out using the Number Cruncher Statistical System (NCSS, Dr J.L.Hintze, Kaysville, UT, USA). Differences between means were tested with the nonparametric Mann–Whitney test and correlation was measured with the nonparametric correlation test according Kendall.

RESULTS

Parasites and vaccination

Based on SDS-PAGE and immunoblotting analysis, the vaccine consisted of a fraction containing almost pure 15

and 24 kDa proteins. There were minor contaminants, predominantly a protein of about 30 kDa (Schallig *et al.* 1997a).

The abomasal worm burdens at week 15 are presented in Table 1. The overall take of the challenge infection in group C was 64% for C3, 34% for C6 and 40% for C9. After vaccination, a significant ($P < 0.01$) reduction of 83% in worm burdens was found in V9 compared with C9. For V6 the reduction was 77% ($P < 0.05$). In contrast, the worm burden of V3 was 34% higher than of C3. As expected, no inhibited larvae were found in any of the abomasa examined.

Group N, the negative group, experienced a negligible infection with a mean worm burden of 20 worms (0.25% of group C9) due to a very low contamination (not shown).

IgE and IgG1 levels

The antibody response against ES following vaccination and challenge infection is given in Figure 1. Only the data of the vaccinated groups are shown because the ODs of the control groups remained below 0.20 for both isotypes until challenge infection. The ODs of the positive control serum on the different plates were within the mean $\pm 15\%$ range. ES-specific IgG1 levels (Figure 1a) were induced in all groups after vaccination and the levels raised after each subsequent vaccination. There was no major difference between the age groups V9, V6 and V3. However, the ES-specific IgE responses (Figure 1b) were very different for the vaccinated age groups V9, V6 and V3. The response in V9 was very strong and a clear booster effect was observed, after each subsequent vaccination the response

Table 1 Total worm counts of the individual sheep of group V and C

V3		C3		V6		C6		V9		C9	
Sheep	Worms	Sheep	Worms	Sheep	Worms	Sheep	Worms	Sheep	Worms	Sheep	Worms
653	7750	650	5700	629	2400	624	6700	523	50	557	7650
654	13950	651	6250	630	1450	627	6150	524	0	560	7800
655	7200	652	7500	631	750	628	7800	526	4700	581	7500
656	5950							529	5350	566	4500
								530	2000	573	12150
								532	0		
								534	150		
								539	50		
								540	150		
Mean	8713		6483		1533 ^a		6883 ^a		1383 ^b		7920 ^b
$\pm$ SE	1786		615		552		560		722		1222
% red	–34		–		77		–		83		–

Mean $\pm$ SE and percentage reduction compared with nonvaccinated sheep of the same age are given. Identical superscripts indicate significant differences; ^a $P < 0.05$ and ^b $P < 0.01$.

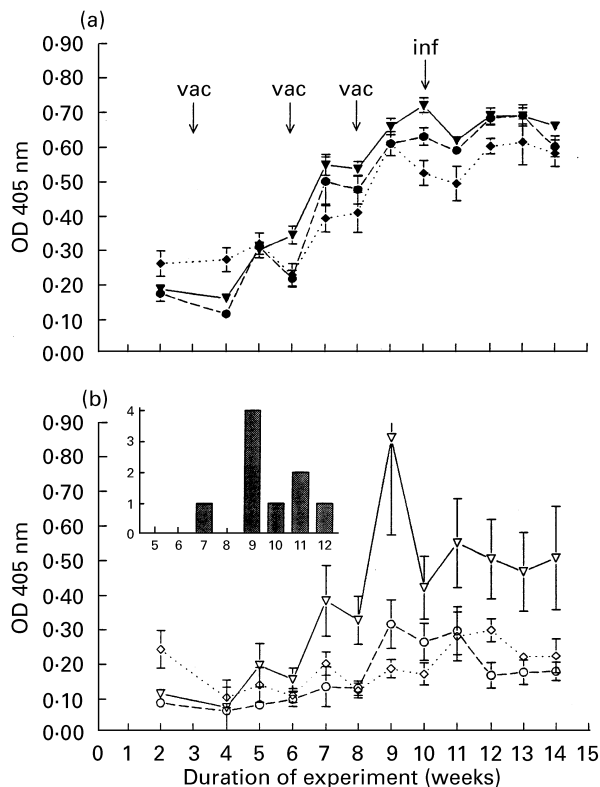


Figure 1 The serum levels (mean $\pm$ SE) of IgG1 (a) and IgE (b) of the groups V9 (∇/∇), V6 ($\bullet/\circ$) and V3 ($\blacklozenge/\diamond$) against ES are given. The time of the three vaccinations (vac) are indicated as well as the time of challenge infection (inf). The insert shows the number of sheep in group V9 that reach their maximum IgE level at the given week of experiment.

became more elevated. Although the highest mean levels are observed 1 week after the last vaccination, the variation in peak levels of individual sheep varied considerably in time (Figure 1b, insert). However, the sheep from group V3 did not show a significant change in ES-specific IgE level during the time of vaccination. The response of V6 was intermediate of V9 and V3, only an increase in IgE levels after the last vaccination was seen. The specific IgE levels of V9 were significantly higher ($P < 0.05$) compared with IgE levels of V3 at week 8 and 9. The ES-specific levels in V6 were significantly higher ($P < 0.05$) compared with the levels of V3 at week 9. The ES-specific IgE, total IgE and IgG1 levels of the individual sera of sheep at the peak of the IgE response (week 9) are given in Figure 2. Here the responses are given as a percentage of the prevaccination values. It should be noted that the variation and the mean values in IgG1 response in all age groups is similar. This in contrast to the ES-specific IgE responses. The variation and mean values for the total IgE levels were low and only slightly greater in V9 than in V3.

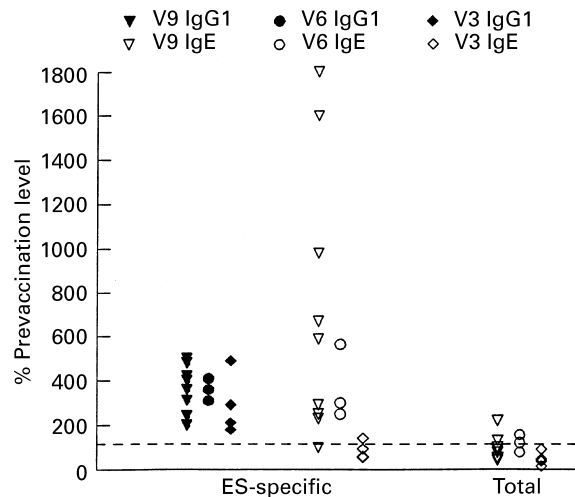


Figure 2 Antibody responses of the vaccinated sheep V9, V6 and V3 1 week after the last vaccination. Serum IgE specific for ES and total IgE (open symbols) and IgG1 specific for ES (closed symbols) were determined. The postvaccination levels of the individual sheep are given here as a percentage of the prevaccination levels (dashed line).

A ES-specific IgE response was not found after vaccination in three out of nine sheep from V9 (sheep 524, 529 and 532) although sheep 524 and 532 were 100% protected by the vaccine, while sheep 529 had the highest worm count of the group. Two animals (sheep 523 and 534) showed a very high IgE level ($>1600\%$ prevaccination) after the third vaccination. They showed, respectively, 99.5% and 98.0% protection and were the only sheep with a clear response after challenge infection. We were not able to find a correlation within group V9 between ES-specific IgE levels and protection, measured as reduction of worm burden. However, when the different age groups V3, V6 and V9 were combined, we found a positive correlation ($P < 0.05$, $r = 0.38$) between protection and ES-specific IgE levels 1 week after the last vaccination. A cluster plot (Figure 3) shows that V3 and V6 form separated clusters with little variation, while V9 shows a large variation in both parameters.

To investigate which ES products were recognized by IgG1 and IgE antibodies, we prepared Western blots of ES products which were probed with sera obtained before and after vaccination (weeks 2 and 9, respectively). Pooled sera of V3, V6 and V9 collected after vaccination and probed with anti-IgG1, showed a similar pattern with similar intensity in all groups. However, the IgE responses differed between the age groups. Pooled sera from V3 and V6 did not contain IgE that recognized ES products, but IgE from V9 recognized several bands on the ES blot (Figure 4a). We therefore decided to reanalyse these sera by probing the ES

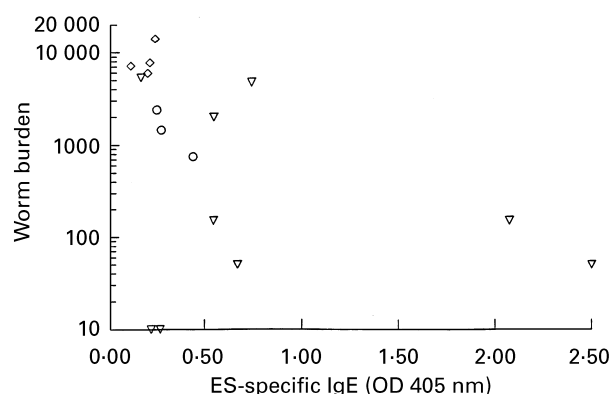


Figure 3 Cluster plot of worm counts versus serum ES-specific IgE levels 1 week after last vaccination. Group V3 ($\diamond$) and V6 ($\circ$) form clusters, V9 (∇) forms a diffuse pattern. Rank correlation coefficient = 0.38.

blots with sera from individual sheep (Figure 4b). The proteins recognized by IgE of the responding sheep from V9 were similar but not identical, all gave reaction with two bands at around 35 kDa and a group of bands at 40–50 kDa. Sheep 524, 530 and 539 also recognized bands of 24 kDa.

Cell counts

At the end of the experiment peripheral blood samples and small abomasal folds were collected, fixed, stained and cell counts were performed to investigate the effect of vaccination and challenge infection. Figure 5 shows the blood eosinophils and eosinophil, SMCP⁺ and mast cell counts in abomasal tissue. Vaccination induced an increased number of mast and SMCP⁺ cells in all age groups. Local eosinophil counts in 3 and 6-month-old sheep were low and vaccination did not alter the local eosinophil counts. In contrast, vaccination did result in an increased number of eosinophils in peripheral blood compared to age matched challenged controls. Furthermore, an age dependent increase in numbers of eosinophils, SMCP⁺ and mast cells was found in the sheep who were vaccinated and challenged. The unvaccinated, unchallenged control group N9 had the lowest cell counts for all three cell types.

DISCUSSION

In this study, we evaluated the effect of vaccination with ES proteins, formulated in DDA, in sheep of different age groups. The three age groups were challenged with different doses. The dose for 3-month-old sheep was 10 000 L3. The 6- and 9-month-old sheep received challenge infections of 20 000 L3, based on their higher body weight. Protection levels were determined by total worm counts and showed

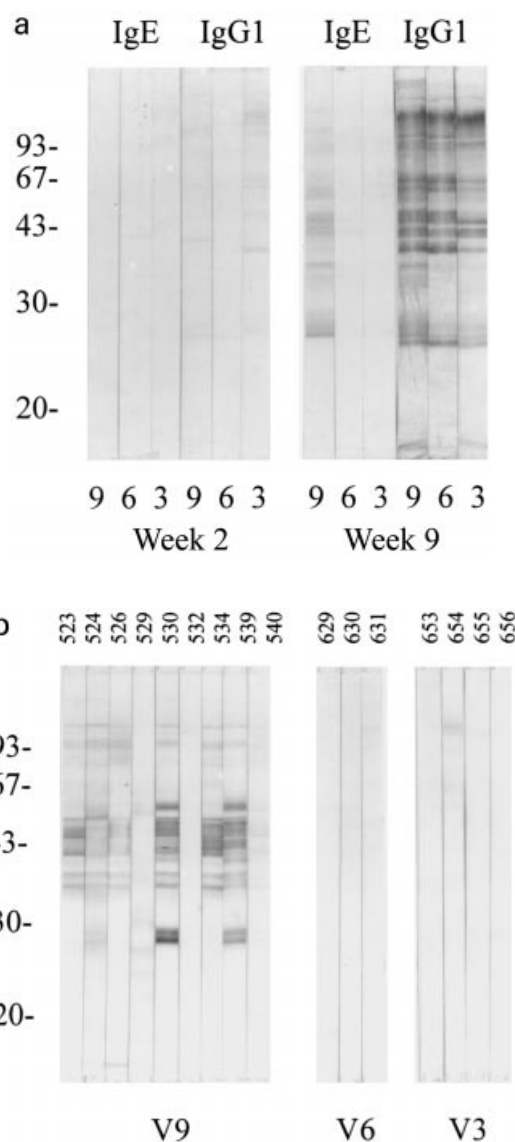


Figure 4 Western blots with ES antigens. Molecular weight markers are indicated at the left. (a) Pooled serum of V9, V6 and V3 at week 2 and week 9 for detection of proteins recognized by IgE or IgG1. (b) Individual sera from V9, V6 and V3 at week 9 for detection of proteins recognized by IgE. Numbers of the individual sheep are indicated above the strips.

that sheep of 6 and 9 months of age were 77% and 83% protected, respectively. In contrast, sheep of 3-month-old were not protected at all by this experimental vaccine (Table 1). We thus set out to correlate these observations with serum levels of IgG1 and IgE, the number of peripheral eosinophils and the number of mast- and SMCP⁺ cells and eosinophils in abomasal tissue.

All vaccinated sheep showed an ES-specific IgG1 response, but only the older sheep showed an ES-specific

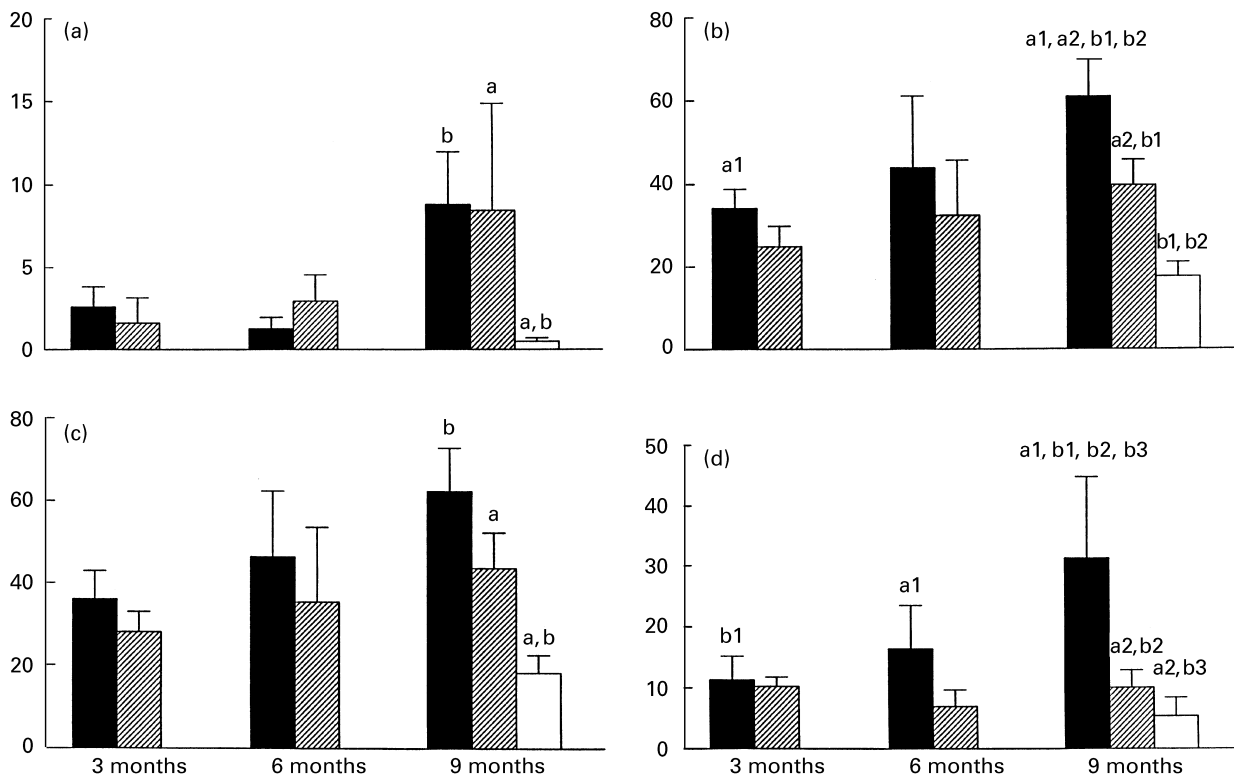


Figure 5 Cell counts (mean/ $0.2 \text{ mm}^2 \pm \text{SE}$) of (a) eosinophils, (b) mast cells and (c) SMCP⁺ cells from abomasal tissue and (d) blood eosinophils (mean per 500 leucocytes $\pm \text{SE}$) at week 15. The age of the subgroups of V (black), C (hatched) and N (white) are given on the x-axis. ^a($P < 0.05$) and ^b($P < 0.01$) indicate significant differences, pairs of identical subscripts indicate significant differences between these pairs.

IgE response (Figures 1 and 2). When all age groups were taken together, there was a positive correlation between ES-specific IgE levels one week after the last vaccination and protection. This correlation may have been stronger if the time interval between the sampling points had been shorter. This is based on two previous observations. First, the individual IgE peaks in a group of sheep, infected with *Teladorsagia circumcincta*, differed with time (Huntley *et al.* 1998). Second, injection of an allergenic *Ascaris* extract in sheep resulted in a narrow peak between day 9–12 post infection (Yilmaz *et al.* 1996). It is also interesting to note here, that vaccination in 9-month-old sheep resulted in elevated specific IgE levels, without increasing the total IgE levels (Figure 2). The specificity of serum IgE was further studied on Western blots (Figure 4). IgE from vaccinated and protected sheep does recognize many ES proteins of around 24, 35 and 40–50 kDa but no protein below 24 kDa (Figure 4b). This may be explained by the recognition by IgE carbohydrate groups on the 24 kDa protein and these groups being present on the contaminating 35 and 40–50 kDa proteins. The 24 kDa protein is known to be glycosylated with one potential N-linked glycosylation

site, whereas the 15 kDa protein does not contain a N-linked glycosylation site (Schallig *et al.* 1997c). It is known that allergens often contain cross-reacting carbohydrate determinants (Koshte *et al.* 1989).

With regard to the observed differences in the number of peripheral eosinophils and the number of eosinophils, mast- and SMCP⁺ cells in the abomasal tissue, we assume that two factors do not affect our observations and conclusions. First, the different doses for the three age groups (10 000 L3 for 3-month-old sheep versus 20 000 L3 for the 6- and 9-month-old sheep) which were compensated for by the difference in body weights. Second, the absence of a negative control for sheep of 3 and 6 months of age. The decision to omit these groups was based on past experience, in which different age groups gave similar base line data for these parameters (unpublished observations). However, in the present study, we found that the number of eosinophils in peripheral blood and the eosinophils, mast- and SMCP⁺ cells in the abomasum increased with age. We also observed that the increase of cell counts in all age groups was positively affected by vaccination, with the exception of the eosinophils in the abomasal tissue. This can point to

a more adequate effector mechanism as sheep mature and become older. Further evidence that an increase in number of eosinophils and mast cells provides a greater degree of protection in sheep has come from recent studies (Pfeffer *et al.* 1996), where one out of four sheep which remained nonimmune to challenge infections, despite repeated infection with *Trichostrongylus axei*, had fewer numbers of tissue eosinophils and IgE positive cells in the abomasum tissue. These findings, taken together with our own, would therefore support a pivotal role for these inflammatory cell reactions in either the development or expression of immune-mediated worm expulsion.

Crucial for the isotype switch to IgE is the presence of IL-4 (Lebman & Coffman 1988). The differences between the groups found in this experiment could be caused by a difference in IL-4 production, in that lack of IgE in young sheep is due to absence or insufficient cytokine production. Insufficient responses in young sheep were also found by Smith & Angus (1980). They showed that two doses of irradiated L3 of *H. contortus* were sufficient to immunize adult sheep while four doses were needed to immunize 2-month-old sheep. In accordance with the present finding, IgG levels were high after immunization and not correlated with protection, while serum IgE was not measured. Tavernor *et al.* (1992) showed that protection of 90% was achieved in 2-month-old sheep by vaccination with the hidden antigen H11 in Quil A. These authors also showed that this protection was based on Ab-mediated inhibition of the aminopeptidase activity of H11 and correlated well with serum IgG levels.

Taken together, the increased serum IgE levels, eosinophilia and mucosal mast cell hyperplasia are the hall marks of a Th2 response and were all demonstrated in protected, older sheep but not in unprotected, young lambs. These data suggest that the differences in protection between young and old sheep after ES-based vaccination are related to IgE and might be based on different thresholds and/or lack of helper signals needed to induce these IgE responses and to recruit the effector cells.

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