

In vitro pre-exposure of *Haemonchus contortus* L3 to blood eosinophils reduces their establishment potential in sheep

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Abstract – Different authors have reported that eosinophils are capable of immobilising infective larvae of different species of nematodes in vitro. However, classifying larvae as mobile or immobile is so subjective that it does not always mean all apparently immobile larvae are dead or those that are mobile are capable of surviving further immune responses if administered to their natural hosts. The objective of this experimental study was therefore to substantiate the role of eosinophils in the killing of *Haemonchus contortus* infective larvae by comparing the infectivity in sheep of larvae that had been incubated with eosinophil-enriched cell suspensions with control larvae. Since it was not possible to isolate pure eosinophils from sheep blood, we were compelled to evaluate the effects of other blood cells contaminating our eosinophil-enriched suspensions. Although eosinophils and neutrophils were the only cells found adherent to *H. contortus* infective larvae in vitro, induced eosinophils in the presence of immune serum were primarily responsible for the drastic reduction in larval motility compared to the minor effects of neutrophils and mononuclear cells. Corresponding reductions in faecal egg count and worm numbers were observed when the incubated larvae were transferred intra-abomasally to sheep. Interestingly, the proportion of larvae that failed to establish was much higher following incubation with induced eosinophils compared with other cells or with immune serum alone. Although this study did not address the in vivo role of eosinophils in sheep, the results strongly indicate that sheep blood eosinophils have a larval killing potential in vitro, and a larval mobility test alone may not fully explain the level of damage inflicted on the larvae.

Haemonchus contortus / eosinophils / in vitro / intra-abomasal / sheep

1. INTRODUCTION

Haemonchus contortus infection in sheep is known to elicit Th2 type im-

mune responses characterised by the recruitment of a large number of eosinophils, mast cells and globule leucocytes and the production of local and circulating antibodies [1, 4, 8, 12]. It has been reported that eosinophils in the presence of immune

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serum were able to immobilise *H. contortus* infective larvae (L3) in vitro [9], but larval killing was suggested only from the observation that the larvae remained immobile for three days. Furthermore, in vivo studies have also described the close association between eosinophils and death of *H. contortus* larvae in the abomasal mucosa of sheep [2]. However, besides the difficulty in classifying larvae as mobile or immobile in an in vitro cell-larvae culture medium, larval motility tests may not always reflect the mortality or inability of those larvae to develop into adult stages if inoculated into their appropriate hosts. Therefore, the present study gives special attention to the capacity of *H. contortus* infective larvae, incubated with blood derived eosinophil-enriched cellular suspensions, to establish in a susceptible sheep. Since eosinophils cannot be completely purified from sheep blood, parallel examinations of the roles of neutrophils and mononuclear cells were also performed.

2. MATERIALS AND METHODS

Four 12 month-old Black belly sheep, raised helminth free indoors, were orally infected with 10 000 *H. contortus* (Humeau strain) infective larvae to become donors of induced eosinophils and immune serum. Blood samples were collected regularly and eosinophils were counted according to the method described previously [3] to judge when a reasonable proportion of eosinophils could be isolated.

The different types of blood leucocytes were isolated using the method adapted from techniques developed by Woldehiwet et al. [15]. Briefly, 30 mL of blood was taken from each Black Belly sheep, on day 15 post infection, in EDTA-coated tubes. After initial centrifugation, the plasma was discarded and the underlying buffy coat layer was care-

fully removed and washed. Only buffy coats with more than 92% mononuclear cells (LYP) were considered for the experiment. Residual red blood cells (RBC) and granulocytes (majority) were processed by haemolysis and subsequent centrifugation to obtain pellets free of RBC. Cells were resuspended in RPMI 1640 (Ref. 21875 GIBCO, Cergy-Pontoise, France) complete medium (supplemented with 1% penicillin-streptomycin and 10% FCS). After centrifugation of the pellets on a percoll density gradient (P1644-Sigma Diagnostics, St. Louis, USA, density: 1.090), cells at the percoll-RPMI interface (EOS) and the bottom-pellet (PMN) were aspirated separately and washed twice with 10 mL cell culture medium. Total and differential cell counts were performed using May Grünwald-Giemsa stain. Pellets containing less than 92% neutrophils were discarded while cells from the RPMI-percoll interface containing more than 40% eosinophils were considered for the in vitro culture. The viability of cells was verified using trypan blue staining. Serum samples were also collected for use in all in vitro tests.

H. contortus L3 were exsheathed using 10 μ L of Milton Sterilising fluid containing 2% w/v sodium hypochlorite and 16% w/v sodium chloride (Milton Pharmaceutical LTD, Thouars, France). After the motility and exsheathment of the larvae were verified, three successive washes with physiological saline solution were performed at low speed centrifugation (250 *g*, 5 min and 20 °C) and the number was adjusted to 1500/mL in a sterile RPMI 1640 complete medium. Cell suspensions were classified as eosinophil-enriched (EOS: from RPMI-percoll interface), neutrophil-enriched (PMN: from the cell pellet) and lymphocyte-enriched (LYP: from the buffy coat layer), and 200 μ L (i.e. 3×10^5 cells/well) of the suspension in RPMI 1640 complete medium were deposited in triplicate in a 24 well test plate

(Ref. 92024, TPP, Geneva, Switzerland) with or without 100 μ L of immune (homologous) whole/decomplemented serum (heat-inactivated at 56 °C, 1 h). In addition, larvae were also incubated with immune serum (SER), and in culture medium only as a control. Finally, 300 exsheathed *H. contortus* L3 in 200 μ L of RPMI 1640 were added and the plates were incubated overnight (18 h) at 37 °C, 5% CO₂.

After 18 h of culture, the motility of 100 larvae was examined directly in the culture plates using an inverted microscope. Larvae were considered mobile if any part of the body was seen moving. An aliquot was taken in a different culture plate from each representative well and stained for three hours using equal volume of eosin Y (Ref. 6766009, Thermo Electron Co., USA) to identify the types of cells (especially eosinophils) adhering to the larvae. Co-cultured larvae were also fixed in glutaraldehyde, dehydrated in ethanol and coated with gold palladium (scanning electron microscopy) or embedded in resin, sectioned and stained (transmission electron microscopy) according to standard procedures for electron microscopic imaging.

After being evaluated *in vitro*, the contents of the culture plates were pooled according to cell types and left overnight at 37 °C mixed with the same volume of distilled water to remove cells. The tubes were then centrifuged at low speed and the number of larvae collected was adjusted to 400 L3/mL in distilled water. Twenty-five four-month old INRA 401 lambs, reared parasite free, were allocated to five groups of five animals. Control lambs (group POS) received 800 non-exsheathed L3 while the remaining four groups received the same number of exsheathed larvae incubated with the following: cellular suspension enriched for induced eosinophils (group EOS), neutrophils (group PMN) or lymphocytes (group LYP) in the presence of immune serum or with serum in a cul-

ture medium without cells (SER). Except for the POS group which was orally infected, direct intra-abomasal injections in lambs of the other four groups were performed under general anaesthesia. Faecal egg counts were performed at regular intervals between days 15 and 28 post infection by the modified McMaster technique [10]. The lambs were killed on day 28 by intravenous barbiturate. Abomasal contents and washings were collected and the worms from a 10% aliquot were counted and classified according to sex and development. The lengths of 20 adult female worms randomly picked from each lamb were measured and the number of eggs *in utero* was determined as previously described [14]. Faecal egg count kinetics were compared between the five groups by analysis of variance with repeated values (SYSTAT software). Comparisons between total worm counts, worm lengths and eggs *in utero* were performed with the non-parametric Kruskal-Wallis test (SYSTAT software) while the proportions of mobile larvae in different culture media were compared using a chi square test.

3. RESULTS

Following *H. contortus* infection of the Black Belly sheep, peak blood eosinophilias were attained between days 9 and 16 post infection (PI). Based on previous observations, day 15 PI was chosen as an appropriate time to isolate induced eosinophils (43–63%). Percentage ranges of neutrophil- and mononuclear cell-enriched suspensions were 92–96% and 92–99% respectively, and viability was between 94 and 96% for all cell types.

Control wells (larvae + culture medium) had 93% actively motile larvae after 18 h of incubation. Eosinophil-enriched cellular suspensions in the presence of immune serum were the most powerful in immobilising the larvae of *H. contortus* ($P < 0.01$)

Table I. Percentages of motile larvae after culture with immune serum and cell suspensions enriched for eosinophils (EOS), neutrophils (PMN) or mononuclear cells (LYP), or with immune serum alone (SER). Values are presented as means and standard deviations for four blood donor animals. Cells from each donor sheep were treated separately for the in vitro tests.

	EOS	PMN	LYP	SER	POS
% Motile larvae (estimated on 100 larvae)	49 ± 4 ^a	74 ± 7 ^b	82 ± 4 ^b	89 ± 4 ^b	–

Values in different letters are statistically different ($P < 0.05$).

followed by neutrophils to some extent and mononuclear cells to a lesser degree (Tab. I). Staining with eosin Y revealed that larvae in the eosinophil- and neutrophil-enriched suspensions were coated with large numbers of cells (Fig. 1A) while those in the mononuclear cell suspensions had very few or no adherent cells (data not shown). Indeed, maximal cell adherence was observed in the first few hours of culture while enhanced larval immobility was seen only after overnight incubation. Lack of further cell adhesion after overnight incubation may indicate cell degeneration or apoptosis. In all cases, heat inactivation of immune serum abolished cell adherence as well as their larval immobilising ability but larval motility was not significantly affected by the presence of serum alone (SER: data not shown). Scanning electron microscopy revealed the close apposition of eosinophils to the larval surface (Fig. 1B) while in transmission electron microscopy, eosinophils had their pseudopodia-like cytoplasmic extensions (Fig. 1C) filled with an electron dense material (Fig. 1D) penetrating into the normally sealed striae of the larval cuticle.

Lambs that received larvae incubated with eosinophil-enriched cells in the presence of immune serum (group EOS) excreted significantly fewer eggs than all the other groups ($P < 0.01$), none of which differed significantly from each other (Fig. 2). Fewer worms (only 12%) were recovered from the EOS lambs compared to the other groups ($P < 0.05$, Tab. II). All recovered worms were adults

Table II. Individual worm counts after intra-abomasal inoculation or oral administration of larvae. Groups EOS, PMN, LYP and SER received exsheathed L3 (800/animal) intra-abomasally while the control group (POS) was infected orally with the same number of larvae. Co-cultured larvae were pooled according to cell type for the intra-abomasal infection.

	EOS	PMN	LYP	SER	POS
Worm counts for each animal	7	760	62	120	475
	136	476	400	633	329
	69	69	405	787	543
	227	640	624	580	513
	45	674	396	378	587
Mean	97 ^a	524 ^b	377 ^b	500 ^b	489 ^b
SD	87	274	132	258	98

Values in different letters are statistically different ($P < 0.05$).

and there was no difference in either female length or number of eggs in utero between groups (data not shown).

4. DISCUSSION

In this study, we were able to obtain 43 to 63% of eosinophils with the major contaminants being lymphocytes. In spite of repeated attempts to optimise the isolation method, we did not attain previously reported levels of purity [15] probably because of the difference in the sheep breed used. Our efforts to isolate eosinophils by the immunomagnetic method [5] have also failed probably due to lack of recognition of sheep neutrophils by human anti-CD16 antibody. However, the 92–99%

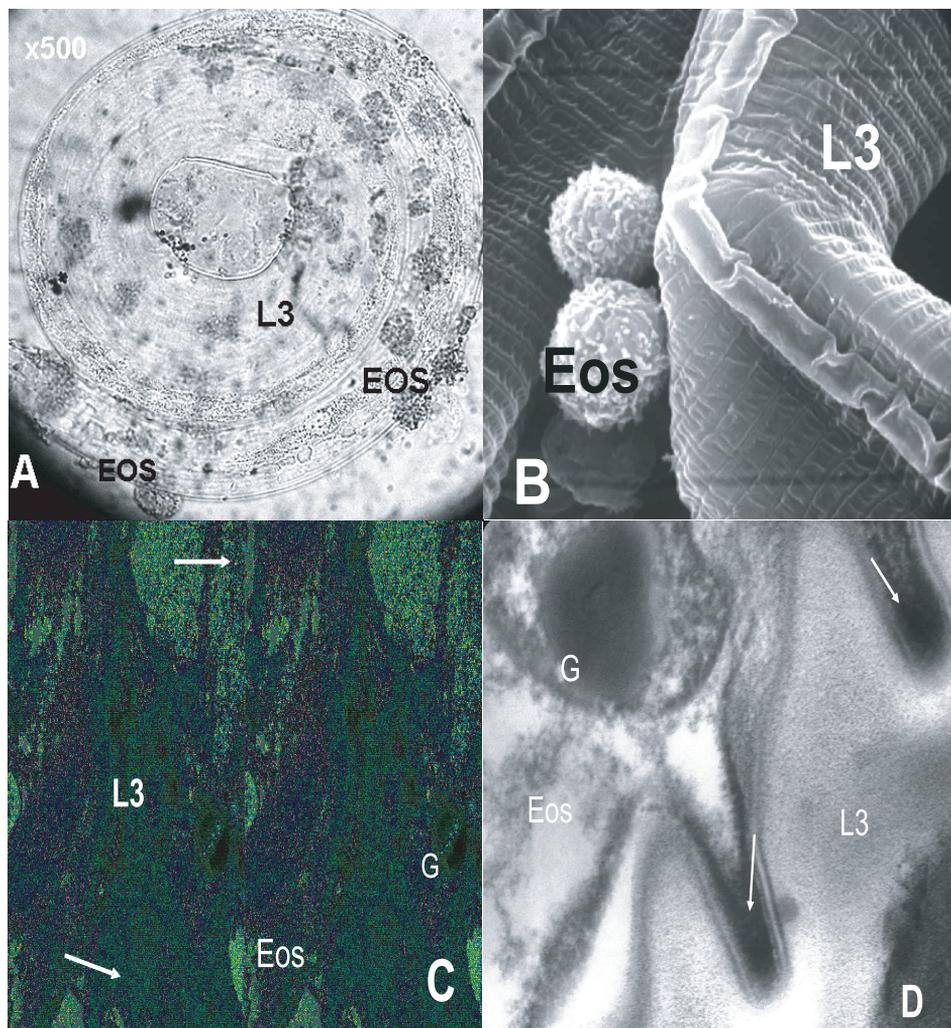


Figure 1. (A) Eosinophils adherent to larvae in eosinophil-enriched culture medium (eosin Y stain). (B) Scanning electron micrograph showing cells apparently eosinophils (Eos) attached to the striated larval surfaces. (C) Transmission electron micrograph showing one eosinophil with partially degranulated granule (G) and cytoplasmic processes penetrating (arrows) into the cuticular striae of the larva. (D) An electron dense material fills the ends of the processes (arrows) in contact with the larval surface.

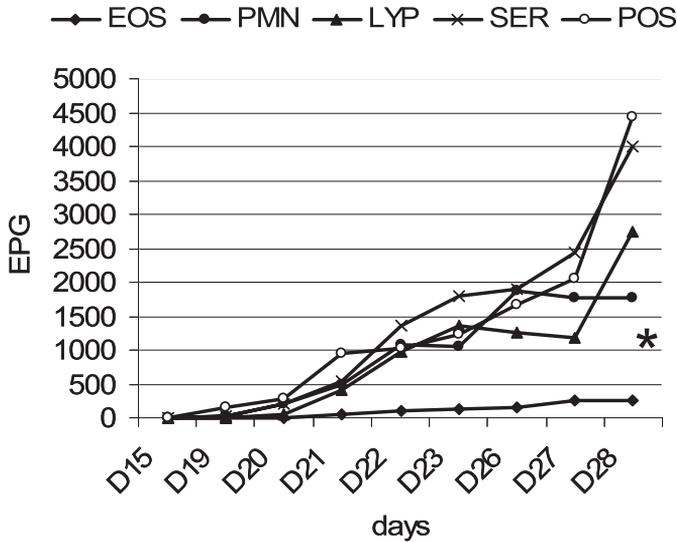


Figure 2. Kinetics of *H. contortus* faecal egg count in four groups of lambs infected by intra-abomasal inoculation of 800 L3 incubated with the following: eosinophil-enriched (EOS), neutrophil enriched (PMN) or mononuclear cell-enriched (LYP) suspensions in the presence of immune serum or with serum alone (SER). A fifth or control group (POS) received the same number of larvae per os. * $P < 0.01$ for group EOS versus other groups.

neutrophils isolated with this technique was close to those found by other authors [15] and the mononuclear cell suspensions were almost free of granulocytes. The motility of larvae incubated for 18 h with eosinophil-enriched suspensions was drastically reduced while the effects of neutrophils and mononuclear cells were much lower. Similar findings were reported using *Strongylus vulgaris* incubated in blood from eosinophilic ponies [7]. The effect of eosinophils on *H. contortus* larval motility was only obvious when immune serum was added and heat inactivation of the serum abolished this effect. Furthermore, as revealed by light and electron microscopy, the eosinophils showed a very intimate contact with these larvae in the presence of immune serum. Although the role of antibody and other serum components cannot be ruled out, it seems very likely that the presence of complement

in the culture was essential for maximum adherence and immobilisation of larvae by eosinophils supporting the findings of Rainbird et al. [9]. Similar in vitro findings were also reported in the study with *Nippostrongylus brasiliensis* in mice [13]. Moreover, by using *Strongyloides stercoralis* L3 in diffusion chambers implanted in immunised mice, Rotman et al. [11] have demonstrated that the majority of the cells recruited towards the larvae are eosinophils and maximal cell number coincides with parasite killing. In this study, it was suggested that direct contact between the cells and the L3 is required for larval killing. Whether this holds true in a classical in vivo condition is not yet clearly demonstrated. Indeed, on the contrary to what has been observed here, there are reports where mice treated with anti-IL-5 mAb exhibit highly reduced numbers of eosinophils but normally reject infections

of helminth parasites [6]. In our study, we also indicate that neutrophils are capable of adhering to the larval surface and hence one can not undermine the contribution of these and other contaminating cells although larval motility was only minimally reduced.

In agreement with our *in vitro* observations, group EOS lambs excreted significantly fewer *H. contortus* eggs and harboured fewer worms than the remaining four groups where any inter-group differences were not significant. Obviously, since female length and number of eggs *in utero* were identical in the five groups, the difference in egg excretion was explained by the difference in total worm burden between groups. Interestingly, most (75%) of the apparently motile eosinophil treated larvae did not establish indicating that eosinophils are far more potent than can be shown by *in vitro* larval motility tests. The larvae were so weakened by the action of eosinophils that they might have been easily expelled before they established.

To our knowledge, this is the first study that combines *in vitro* larval immobilisation tests with the subsequent *in vivo* development of the same larvae in sheep. Although this study provides no data on the *in vivo* role of eosinophils, the *in vivo* experiment not only confirmed our *in vitro* observations but also demonstrated that the majority of those larvae that were considered mobile (alive) *in vitro* were incapable of surviving further in the host.

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