

Stimulation of the *In Vitro* Migration of Ovine Eosinophils by Factors Derived from the Sheep Scab Mite, *Psoroptes ovis*

L.A. Wildblood and D.G. Jones*

Moredun Research Institute, Pentlands Science Park, Penicuik, Scotland, UK

*Correspondence: E-mail: Doug.Jones@moredun.ac.uk

ABSTRACT

The ectoparasitic astigmatid mite *Psoroptes ovis* causes sheep scab, a highly contagious, severe allergic dermatitis associated with damage to the fleece and hide, loss of condition and occasional mortality. The scab lesion is characterized by a massive infiltration of eosinophils that begins very rapidly after infection. This paper reports the finding that mite-derived factors directly enhance the migration of ovine eosinophils *in vitro*. Significant ($p < 0.01$) and dose-dependent ($r = 0.972 \pm 0.018$ (SD)) activity was initially identified in whole mite extracts, by comparison with medium controls in an assay based on modified Boyden chambers and ovine bone marrow target cells. Similar pro-migratory activity ($p < 0.005$; $r = 0.928 \pm 0.069$ (SD)) was detected in washes containing mite excretory/secretory material. By direct comparison with migration ratios ($n = 3$) for defined chemotactic (rmeotaxin = 3.430 ± 0.360 (SD)) and chemokinetic (rminterleukin-5 = 0.982 ± 0.112 (SD)) stimuli it was determined that the activity in both mite extracts (0.992 ± 0.038 (SD)) and mite washes (0.969 ± 0.071 (SD)) was chemokinetic. Subsequent experiments ($n = 3$) in which live mites were incorporated directly into the *in vitro* assay system indicated that they produced factors that significantly ($p < 0.001$) enhanced eosinophil migration to a degree directly related to mite numbers ($r = 0.993 \pm 0.005$ (SD)). The identity of the factor(s) responsible is uncertain, but their presence suggests that mites may be capable of directly activating eosinophils *in vivo*, and raises the possibility that mites could directly influence, perhaps even initiate, the rapid early tissue eosinophilic response observed in experimental sheep scab infections.

Keywords: ectoparasites, eosinophils, excretory/secretory products, *in vitro* chemotaxis/chemokinesis, mites, *Psoroptes ovis*, sheep scab

Abbreviations: FCS, fetal calf serum; HBSS, Hanks balanced salt solution; IL-5, interleukin-5; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline; r , correlation coefficient; rm, recombinant murine; SBMC, sheep bone marrow cells; SD, standard deviation; SDS, sodium dodecyl sulphate; SEM, standard error of the mean

INTRODUCTION

Sheep scab, caused by the astigmatid mite, *Psoroptes ovis*, poses a severe welfare problem. Infestation by this obligate, non-burrowing ectoparasite provokes a severe exudative skin lesion associated with intense pruritis, which leads to rubbing, scratching, wool loss, wounding and fitting (Bates, 1997; van den Broek and Huntley, 2003). The condition has all the hallmarks of an allergic dermatitis and this is endorsed by the classic immediate-type hypersensitivity reaction evoked by intradermal injection of mite antigen (Wikel, 1984; Pruett *et al.*, 1998). A feature of the infection site is the rapid (within 24 h) and massive infiltration of eosinophils, which form the predominant cellular component of the characteristic scab lesion (van den Broek *et al.*, 2000; van den Broek and Huntley, 2003). The rapid onset of the tissue eosinophilic response, allied to observations that the mites appear to rely on ingestion of serous exudate from the advancing lesion as a

primary nutrient source (Mathieson, 1995; Beetham, 1997; Pettit *et al.*, 2000), and the fact that the presence of large numbers of eosinophils does not prevent expansion of the lesion or the increase in mite numbers, has led to speculation in this laboratory that *P. ovis* may be directly involved in the recruitment and/or function of these cells as a means to promote their own maintenance and survival. Support for this hypothesis emerged from a study in which administration of an anti-inflammatory corticosteroid (dexamethasone) transiently impaired development of the lesion and depressed mite numbers (Bellworthy *et al.*, 1997).

The main objective of the present studies was to ascertain whether sheep scab mites contain and/or produce factors that might directly influence the migration of ovine eosinophils, using an *in vitro* assay technique based on modified Boyden chambers and sheep bone marrow-derived eosinophils as target cells. A subsequent aim was to characterize the nature of any migratory responses demonstrated by reference to eotaxin and interleukin-5 (IL-5), previously shown to selectively induce eosinophil chemotaxis (Jose *et al.*, 1994) and chemokinesis (Palframan *et al.*, 1998), respectively.

MATERIALS AND METHODS

Reagents

Recombinant murine (rm) interleukin-5 (IL-5) and eotaxin were purchased from Sigma Ltd, Poole, UK. For use as positive controls, rmIL-5 and rmeotaxin were diluted in Iscove's modified Dulbecco's medium (IMDM; Sigma) supplemented with 10% fetal calf serum (FCS), 0.05 mmol/L 2-mercaptoethanol (Sigma), 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco BRL, Paisley, UK) and 10 IU/ml preservative-free heparin (Sigma).

Mite donor animals

P. ovis-naïve crossbred sheep, aged 1–2 years, were infested on the withers with a 10 mm biopsy punch of mite-infested skin from a previously infected donor animal. The animals were left for 6–7 weeks, then euthanized by lethal injection. Mites were harvested, as a natural mixed developmental stage population, from skin sections taken from the lesion area at postmortem by placing them in Petri dishes under electric lamps and leaving them overnight (or longer) to allow migration of the mites to the Petri dish lid. For migration experiments with live mites, the lids were placed on an inverted light microscope, and mites were transferred individually into the lower compartment of the Boyden chambers used.

Preparation of whole mite extract

Approximately 10 ml of ice-cold phosphate-buffered saline, pH 7.4 (PBS) was added to 1 ml of packed frozen, mixed stage, *P. ovis* mites and vigorously agitated for 5 min, then centrifuged for 5 min at approximately 12 000g. After removal of the supernatant, the pellet

was resuspended in 1% sodium dodecyl sulphate (SDS), then agitated and centrifuged as above. The pellet was washed 10 times in ice-cold PBS, resuspended in 10 ml of ice-cold PBS, homogenized on ice, then sonicated 5 times for 1 min. The homogenization step was repeated and, after centrifugation at approximately 1500g for 10 min, the supernatant was retained as 'whole mite extract'.

Mite wash preparations

A 1:1 (v/v), mixture of mites with ice-cold PBS was vortexed for 5 min, then centrifuged at 12 000g for 5 min. The resulting supernatant was retained as 'mite wash'.

Collection and preparation of sheep bone marrow cells (SBMC)

A variety of crossbred lambs, under 1 year of age, of either sex, were used as bone marrow donors. SBMC were isolated from sternal marrow obtained at autopsy and prepared as previously described (Haig *et al.*, 1988). Briefly, each marrow cell suspension was filtered through two thicknesses of lens tissue (Whatman UK Ltd, Maidstone, UK) to remove fat and tissue and the filtered cells were then washed in Hanks balanced salt solution (HBSS; Invitrogen Life Technologies Ltd, Renfrew, UK) containing 2% heat-inactivated FCS (Sigma), heparin, penicillin and streptomycin (see above) and 8% sodium bicarbonate (Fisher Scientific Ltd, Loughborough, UK). After resuspending the cells in HBSS medium, a fraction of density < 1.077 g/ml was obtained by centrifugation over Lymphoprep (Nycomed UK Ltd, Oxford, UK) at 1000g for 30 min at room temperature. After further washing in HBSS medium, the interface cells were adjusted to 5×10^6 viable, nucleated cells/ml in 10% IMDM for use in the migration assay. Differential eosinophil cell counts in blood and bone marrow were performed using freshly prepared Carpentier's stain (1:50 dilution of 2% eosin Y (Sigma) and 3% of 37% formaldehyde saturated with calcium carbonate). Cells were diluted 1:10 and the percentage of eosinophils was determined by light microscopy ($\times 20$ objective) in modified Neubauer chambers.

Eosinophil migration assay

Eosinophil migration was measured in blind-well Boyden chambers (NeuroProbe Inc., Gaithersburg, MD, USA). Briefly, the lower compartment was filled with either rmIL-5 or rmeotaxin (100 ng/ml as positive controls for mite preparation experiments), whole mite extract or mite wash, diluted in 10% IMDM and covered with an 8 μ m polycarbonate filter (Sartorius UK Ltd, Epsom, UK). 10% IMDM alone was used as a negative control. To distinguish between chemotaxis and chemokinesis, chambers were also set up containing protein equivalent concentrations of positive controls or test samples in both the upper and lower compartments. For live mite experiments the required number of mites, randomly selected from the harvested mixed-stage populations, were introduced to the lower compartment, which was then filled with 10% IMDM before covering with polycarbonate membrane. SBMC (200 μ l) at 5×10^6 /ml was added to the upper compartment and the chambers

were then incubated for 3 h at 37°C, 5% CO₂. After incubation, the filters were removed, washed in PBS, then fixed overnight in 70% ethanol (Fisher Scientific). The fixed filters were differentially stained using Diff-Quik (Baxter Diagnostics, McGraw Park, IL, USA), then dehydrated and cleared stepwise in ethanol (Fisher Scientific), *n*-propanol (Rathburn Chemicals Ltd, Walkerburn, UK), *n*-propanol–xylene (1:1 v/v) and xylene (Fisher Scientific) and then mounted onto microscope slides. The number of stained cells in five randomly chosen fields of view was determined by light microscopy ($\times 40$) at a depth of 50 μ m from the upper surface of the filter.

Total protein measurement

Total protein concentrations in the various mite preparations were estimated using a commercial kit (Pierce BCA reagent, Perbio Science, Cramlington, UK).

Statistical analysis

Significance was tested against the negative (medium only) control by ANOVA or Student's *t*-test with a probability (*p*) <0.05 being accepted as a significant difference. Linearity of responses was evaluated by regression analysis. Associated correlation coefficients (*r*) >0.9 were taken as indicative of linearity.

RESULTS

rmIL-5 and rmeotaxin dose–response curves

Both rmIL-5 and rmeotaxin, when added to the lower chamber only, stimulated reproducible and dose-dependent migratory responses from ovine bone marrow-derived eosinophils in the *in vitro* assay procedure used (data not shown). Both recombinants induced a linear response at concentrations between 25 and 200 ng/ml (correlation coefficients *r* = 0.965 and *r* = 0.975 for rmIL-5 and rmeotaxin, respectively), and were stimulatory at concentrations down to 10 ng/ml. The rmeotaxin continued to give a linear response up to at least 400 ng/ml, but at that concentration the response to rmIL-5 had reached a plateau. In subsequent studies investigating the migratory responses of mite products, a concentration of 100 ng/ml (which stimulated a response approximately 3 times greater than the IMDM control) was adopted as a positive control for both rmIL-5 and rmeotaxin. In order to differentiate between chemotactic and chemokinetic responses migratory responses were compared in experiments in which 100 ng/ml rmIL-5 or rmeotaxin was added to the lower compartment only, or both upper and lower compartments, of the Boyden chambers. Data from five separate experiments are shown in Table I. Migration was largely unaffected when equal concentrations of rmIL-5 were present in both compartments, but was almost completely abrogated when rmeotaxin was added to both chambers. In numerical terms, based on the observed ratio of the migratory response to the stimulus in the lower compartment only (L+ U–) to the response to the same concentration of stimulus in upper and lower compartments (L+ U+), 'migration ratios' close to 1 indicated a chemokinetic response, while values >2 indicated a

TABLE I

Comparison of chemotactic (rmeotaxin) and chemokinetic (rmIL-5) response patterns of ovine eosinophils *in vitro*

Experiment	IMDM	rmIL-5			rmeotaxin		
		L+ U–	L+ U+	Ratio ^a	L+ U–	L+ U+	Ratio ^a
1	34	103	105	0.981	89	38	2.342
2	19	87	95	0.916	82	16	5.125
3	23	93	95	0.979	83	25	3.320
4	21	92	84	1.095	91	29	3.138
5	8	61	70	0.871	46	12	3.833
Mean	21.00	87.20	89.80	0.968	78.20	24.00	3.552
SD	9.30	15.75	13.33	0.084	18.40	10.37	1.030

^aRatio = (L+ U–/L+ U+). L = lower compartment and U = upper compartment of Boyden chamber

chemotactic response. These numerical criteria were used to qualitatively assess the nature of responses to mite products.

Whole P. ovis mite extract

In a series of preliminary experiments ($n = 5$), it was established that whole mite extracts, adjusted to total protein concentrations of 5, 10 and 20 $\mu\text{g/ml}$, stimulated a significant ($p < 0.01$) and linear ($r = 0.972 \pm 0.018$ (SD)) dose-dependent eosinophil migratory response (Figure 1a). Figure 2 shows a summary of three subsequent experiments aimed at evaluating the nature of this migratory response at a single whole mite extract total protein concentration of 10 $\mu\text{g/ml}$. For these three experiments, the mean $\pm$ SD migration ratios for rmIL-5 and rmeotaxin were 0.982 ± 0.112 and 3.430 ± 0.360 , respectively. The corresponding mean $\pm$ SD migration ratio for whole mite extract was 0.992 ± 0.038 . This was significantly ($p < 0.01$) lower than for rmeotaxin and very similar to the value for rmIL-5.

P. ovis mite wash

Mite washings ($n = 5$), adjusted to the same total protein concentration as the whole mite extracts (see Figure 1b), stimulated a similar, even marginally more potent ($p < 0.005$), migratory response compared with the extracts. The response was linear ($r = 0.928 \pm 0.069$ (SD)) over the mite wash total protein range 5–20 $\mu\text{g/ml}$. Three batches of mite wash tested in subsequent experiments gave a mean $\pm$ SD migration ratio 0.969 ± 0.071 .

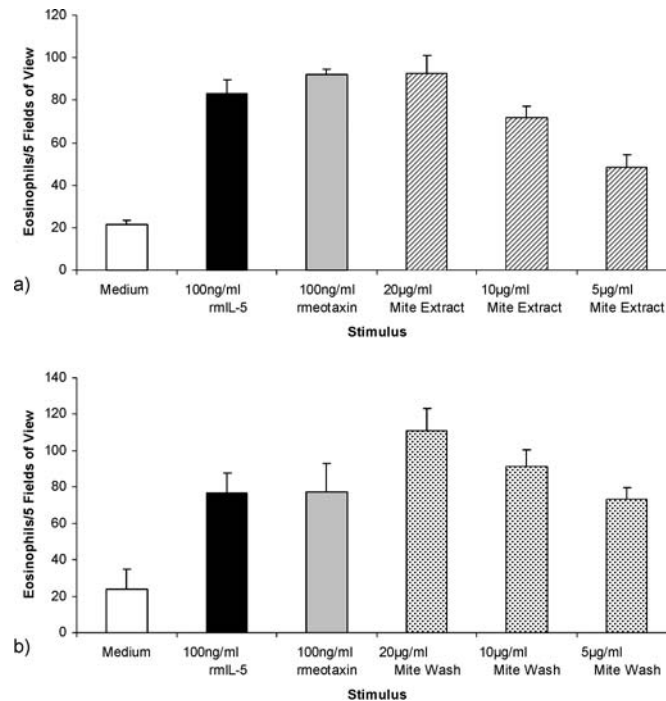


Figure 1. Mean (+ SEM error bars, $n = 5$) total number of eosinophils counted by light microscopy ($\times 40$ objective, at $50 \mu\text{m}$ from the upper surface), in five separate fields of view in stained filters from Boyden chambers containing either medium alone, 100 ng/ml rmIL-5, 100 ng/ml rmeotaxin and (a) whole mite extracts or (b) mite wash at 20, 10 and $5 \mu\text{g/ml}$ total protein content, in the lower compartment as stimulant

Live P. ovis mites

As illustrated in Figure 3, which shows the mean results from three separate experiments using mites harvested from three separate groups of donor animals, the presence of as few as 10 live mites, incorporated directly into the medium in the lower compartment of Boyden chambers used for the chemotaxis assay, induced a chemotactic response significantly ($p < 0.001$) greater than that observed with medium alone, and the potency of the response was approaching that induced by the rmIL-5 positive control. The response increased in a linear fashion ($r = 0.993 \pm 0.005$ (SD)) when up to 50 live mites were added.

DISCUSSION

This study utilized an *in vitro* assay procedure, based on modified Boyden chambers, specifically adapted to evaluate chemotactic responses of ovine eosinophils isolated from fresh bone marrow preparations. An initial requirement was to establish reliable positive controls

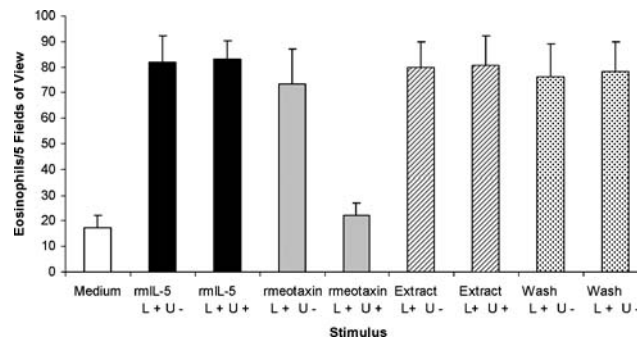


Figure 2. The results from three separate experiments in which 100 ng/ml rmIL-5, 100 ng/ml rmeotaxin, whole mite extract or mite wash (both at 10 μ g/ml total protein content), were added either to the lower compartment only (L+ U-), or to both upper and lower compartments (L+ U+), prior to incubation. The figures are the mean ($\pm$ SEM error bars) total numbers of eosinophils counted by light microscopy ($\times 40$ objective, at 50 μ m from the upper surface), in five separate fields of view in stained Boyden chamber filters

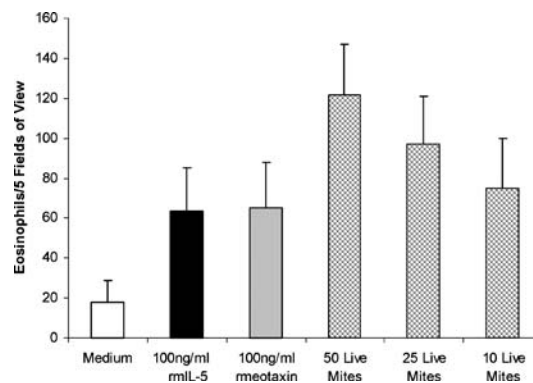


Figure 3. Mean ($\pm$ SEM error bars, $n = 3$) total number of eosinophils counted, by light microscopy ($\times 40$ objective, at 50 μ m from the upper surface), in five separate fields of view in stained chemotaxis filters from chambers containing medium alone, 100 ng/ml rmIL-5, 100 ng/ml rmeotaxin and medium containing 50, 25 or 10 live mites, harvested from separate donor animals, as stimulant

for the assay, which could also distinguish between chemokinetic and chemotactic activity. In this regard rmIL-5 and rmeotaxin were both shown to stimulate a linear dose-responsive eosinophil migratory response in the routine assay (data not shown). In subsequent experiments with mite products, rmIL-5 and rmeotaxin, each at a concentration 100 ng/ml, were used as routine positive controls. At this concentration, the activity of rmeotaxin, a known chemotaxin (Jose *et al.*, 1994), but not rmIL-5, an eosinophil activator with chemokinetic properties (Palframan *et al.*, 1998), was abrogated when added to both Boyden chamber compartments (see Table D). Human and murine IL-5 recombinants have previously been reported to exhibit species cross-reactivity in a study using an enzyme-based ovine eosinophil

proliferation/survival assay (Stevenson and Jones, 1994). However, as far as the authors are aware, this is the first description of functional species cross-reactivity for eotaxin.

Initial experiments identified that crude whole mite extracts induced potent and dose-dependent eosinophil migration *in vitro* (Figure 1a). Moreover, results from a subsequent *in vivo* pilot experiment (A.H. van den Broek, unpublished observation) indicated that intradermal inoculation of similar crude extracts was associated with a marked accumulation of eosinophils at the injection site within 24 h. However, neither of these studies provided definitive evidence that the mites actively produced chemoattractant activity, and the observations may have been due to the presence of constitutive endogenous mite components. More compelling *in vitro* evidence was obtained firstly by showing that mite washings also contained potent pro-migratory activity (Figure 1b) and secondly by incorporating washed, live mites directly into the Boyden chambers, and demonstrating, on repeated occasions, that their presence also stimulated a potent eosinophil migratory response, the magnitude of which was directly related to the number of mites present (Figure 3). The observations with both live mites and mite washings suggested that the stimulatory factor(s) were excretory-secretory products, although the possibility that they were from other sources, e.g. damaged mite body parts or contaminating non-mite material such as mite-associated bacteria, cannot be excluded.

Although the initial experiments demonstrated the dose-dependence of the migratory response, they did not describe the nature of the response. In a further series of experiments using defined controls, it was established that the effect of mite-derived products was chemokinetic rather than chemoattractive (Figure 2). In other words, the activity more closely resembled, that of IL-5 than that of eotaxin. Interestingly in this respect, although the identity of the mite-derived chemokinetic activity remains unknown at present, it has recently been shown that a monoclonal antibody that completely neutralized the activity of rmlL-5 partially reduced (by approximately 40%) the activity of whole mite extracts (L.A. Wildblood and D.G. Jones, unpublished observation). In contrast, in the same studies, neither a second monoclonal antibody that abolished rmeotaxin activity or a control rabbit IgG2 had any significant effects on mite-derived activity.

The current results add to the continuing enigma surrounding the physiological function(s) of eosinophils and, in particular, the debate on their role(s) in parasitic infection. Eosinophils are undoubtedly active in producing the pathology of inflammation and in perpetuating the inflammatory response in allergic diseases (Martin *et al.*, 1996; Palframan *et al.*, 1998), although there is now evidence that eosinophils contribute to tissue repair as well as injury (Munitz and Levi-Schaffer, 2004; Williams, 2004). For parasitic diseases, mostly in the context of rodent models of helminth infection, there is some evidence that eosinophils may mediate killing *in vivo* (Meeusen and Balic, 2000) as well as *in vitro* (Butterworth, 1984; Butterworth and Thorne, 1993; Rainbird *et al.*, 1998), although there are other indications that eosinophils may act protectively or deleteriously depending on the circumstance (Dombrowicz and Capron, 2001; Klion and Nutman, 2004).

In terms of ectoparasites, such as *P. ovis*, there is little to suggest that the massive dermal eosinophilic response underpinning lesion formation is of advantage to the host, or destructive to the mite. Indeed, the response appears to benefit the parasite by providing the serous exudate on which the mites feed and thrive (Mathieson, 1995; Beetham, 1997; Pettit *et al.*, 2000). The present study provides *in vitro* evidence suggesting that mites actively produce

factor(s) that induce eosinophil chemokinesis. The latter is indicative of cell activation and indicate that mites could directly influence both recruitment and function *in vivo*. Tissue damage caused by activated eosinophils and their armoury of potent inflammatory effector molecules (Walsh, 1999) could then contribute to the formation of the scab lesion, and this might provide a skin microenvironment conducive to mite maintenance and survival.

Ongoing work is attempting to fully characterize the chemokinetic factor(s) described in order to explore their possible potential as therapeutic targets. Strategies aimed at damping the host eosinophilic inflammatory response might also offer novel immunotherapeutic approaches to mite control.

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