# Integrin-like RGD-dependent cell adhesion mechanism is involved in the rapid killing of *Onchocerca* microfilariae during early infection of *Simulium damnosum* s.l.

H. E. HAGEN[1,2]* *and* S. L. KLÄGER[2]

[1] *Department of Biological Sciences, Keele University, Staffs ST5 5BG, UK*
[2] *School of Environmental and Life Sciences, Salford University, Salford M5 4WT, UK*

(*Received 7 August 2000; revised 7 October 2000; accepted 11 October 2000*)

SUMMARY

Injection trials with compatible and non-compatible *Onchocerca* species into *S. damnosum* s.l., the vector of human and bovine onchocerciasis, demonstrated that the rapid killing of microfilariae within the blackfly's haemocoel is species specific. In the presence of the peptide RGDS as a blocking agent for integrin-like receptors of haemocytes, the survival of *O. ochengi* microfilariae in its natural intermediate host was significantly increased. This increased survival 24 h p.i. correlated with a significant decrease of apoptosis levels in the microfilariae following a 2 h exposure to the haemolymph *in vivo*. These findings suggest that haemocytes are directly involved in the killing of *Onchocerca* microfilariae in the blackfly.

Key words: innate immunity, haemocytes, apoptosis, cytotoxicity, natural killer cells, *Simulium damnosum*, *Onchocerca ochengi*, blackflies, filarial infection, river blindness.

## INTRODUCTION

Blackflies of the *Simulium damnosum* species complex are the vectors of *Onchocerca volvulus*, the cause of river blindness affecting around 20 million people world-wide (WHO, 1995). As it is too difficult to obtain sufficient *O. volvulus* material for infection trials we have established a model system to investigate the role of the innate immune system of *S. damnosum* s.l. for the transmission of human river blindness using *O. ochengi*, a bovine parasite (Bwangamoi, 1969). This species is naturally transmitted by the same vector as *O. volvulus* and is its closest relative (Denke & Bain, 1978). Using microfilariae of a temperate *Onchocerca* species, *O. lienalis*, and a North American blackfly, *Simulium vittatum*, it could be demonstrated that a certain proportion of microfilariae that reach the blackfly's haemocoel will be killed very rapidly within the first 2 h (Lehmann, Cupp & Cupp, 1994). Most of the surviving microfilariae will continue to develop successfully to infectious larvae, and it is uncommon to find dead or damaged microfilariae, but the ones that are found show multiple sites of damage. This '*lytic*' response was interpreted as unique for blackflies at the time. The lytic effect could be saturated by re-infecting blackflies with microfilariae demonstrating that there was only a limiting amount of this factor available. Possible active factors of this response to filarial infection could belong to the blackfly's innate immune system. What is somewhat unique for adult blackflies is the absence of encapsulation and melanization reactions against intruding particles and pathogens, although important pathways – like the phenoloxidase pathways – are intact (Hagen, Grunewald & Ham, 1994; Hagen *et al*. 1997*a*). The humoral arm of the immune response of *S. ornatum*, a European species, and *S. damnosum* s.l. has been extensively investigated (Ham *et al*. 1995), and it could be demonstrated that as in other insects a range of molecules are implicated in the response to infection (Richman & Kafatos, 1995). But very often direct evidence of the activity of immune molecules against *Onchocerca* parasites was missing although it had been demonstrated that certain undefined components of immune haemolymph of blackflies led to an enhanced killing of the parasite (Ham, 1986). To assess if and which components of the innate immune system might be responsible for the killing of microfilariae, it was of interest to determine how exactly the parasite dies after entering the haemocoel of the vector. It was recently demonstrated that the microfilariae showed enhanced apoptosis levels when exposed to the haemocoel of blackflies by using caspase inhibitors in co-injection trials and a cell death detection assay (Hagen, Kläger & Williams, 1998). In addition, it could be shown in these *in vivo* experiments that the onset of apoptosis was potentially mediated by trypsin-like proteases (Hagen *et al*. 1998), known to be transcriptionally up-regulated in the vector during an *Onchocerca* infection (Hagen *et al*. 1995*a*; Hagen, unpublished

* Corresponding author: International Programme, The Wellcome Trust, 183 Euston Road, London NW1 2BE, UK. Tel: +020 7611 8203. Fax: +020 7611 8373. E-mail: h.hagen@wellcome.ac.uk

 
https://doi.org/10.1017/S0031182001007545 Published online by Cambridge University Press
DOI: 10.1017/S0031182001007545 Printed in the United Kingdom

observations). Co-injections of caspase as well as serine protease inhibitors together with microfilariae resulted in a significantly enhanced survival of the parasites (Hagen *et al*. 1997*b*, 1998). However, it was not clear which part of the immune system was responsible for the enhanced levels of apoptosis. This present study was aimed to investigate whether or not haemocytes, e.g. the cellular arm of the immune system, were involved. However, very little is known about the role of blackfly haemocytes and the body of publication is very small. Cupp, Chen & Cupp (1997) demonstrated shifts in the haemocyte populations in sham-inoculated and *Onchocerca*-infected *S. vittatum* compared to controls. But this could be interpreted as a general stress response rather than a specific reaction to infection. As it proved difficult to maintain blackfly haemocytes *in vitro*, especially under field conditions, it was necessary to find an inhibitor that could be used to interfere with haemocyte adhesion to microfilariae *in vivo*. Integrins are known to be important receptors for haemocyte adhesion (Johansson, 1999). Recently, Pech & Strand (1995), using a peptide (RGDS) mimicking the common binding motif of integrins, demonstrated that haemocyte adhesion in the moth *Pseudopleusia includens* was RGD dependent. We therefore used this peptide as a tool to investigate whether or not haemocytes would have any effects on microfilarial survival of *O. ochengi* and their apoptosis levels during early *in vivo* exposure to haemolymph of *S. damnosum*.

## MATERIALS AND METHODS

### *Blackfly and parasite material*

Female *S. damnosum* s.l. were collected as pupae on trailing vegetation from the Vina du Sud, near the town of Ngaoundéré, Adamawa Province, Cameroon. The blackflies were kept in specially designed cages (Wenk & Raybould, 1972). Only females were used for the injection experiments within 2 days of their emergence. The microfilariae of *O. ochengi*, *O. dukei* and *O. gutturosa* were isolated from bovine skin samples collected at the local abattoir of Ngaoundéré. The microfilariae were isolated following the method described by Bianco *et al*. (1980) and purified following the method of Hagen, Kläger & Ham (1995*b*). While *O. ochengi* develops in *S. damnosum* s.l. the other two species do not (Wahl, Ekale & Schmitz, 1998). RPMI 1640, supplemented with 200 U/ml penicillin, 200 $\mu$g/ml streptomycin and 100 $\mu$g/ml Nystatin, was used as medium for isolation of microfilariae and during the injection trials.

### *Mode of infection*

The *Onchocerca* species were identified morphologically (Bain *et al*. 1977). Flies were inoculated through their pleural membrane using drawn out glass capillaries (Nelson, 1962). The flies were kept on a 30 % sugar solution containing penicillin and streptomycin (200 U/ml and 200 $\mu$g/ml respectively) and Nipagin (0·25 %).

*Differential injection trials*. Findings by Lehmann *et al*. (1994) suggested that most *O. lienalis* microfilariae were killed during the first 2 h following injection into the North American blackfly species *S. vittatum*. These present trials were conducted to ascertain whether or not the optimal *in vivo* incubation time of *O. ochengi* microfilariae in the haemolymph of *S. damnosum* s.l. for the TUNEL assays was also 2 h. Female *S. damnosum* were inoculated with 15 microfilariae of 1 of 3 bovine *Onchocerca* species: the compatible species *O. ochengi*, and the 2 non-compatible species *O. dukei* and and *O. gutturosa*. The blackflies were dissected after 2 h and the microfilariae counted.

*RGDS/RGES injection trials*. (A) The effects of haemocytes on the survival of microfilariae were investigated by co-injecting *O. ochengi* microfilariae with RGDS (Sigma A9041), the blocking agent for integrin-like receptors on haemocytes. Two control groups were included in the experiment (i) the peptide RGES (Sigma A5686) has no blocking properties due to the change of 1 amino acid and was used at the same molarity, (ii) plain medium. Graded glass capillaries were filled with 0·25 $\mu$l of 40 mM RGDS or RGES peptide solution or plain medium, followed by the uptake of 5 microfilariae from the microfilariae suspension. The injection volume was adjusted to a total of 0·5 $\mu$l with plain medium if necessary. The final concentration of peptides within the blackflies was estimated to be 10 mM. For each experimental group, 50 flies were injected, and the surviving flies were dissected 24 h later and the microfilariae were counted.

(B) To investigate the effects of haemocytes on levels of apoptosis, blackflies were inoculated with 15 microfilariae of *O. ochengi* together with RGES (30 flies), or with RGDS (30 flies). The experimental conditions were the same as described for the survival trials above. In this case the blackflies were dissected at the earlier time point of 2 h p.i. and the microfilariae were retrieved with drawn-out glass capillaries and stored in liquid nitrogen for the following *in situ* cell death detection assay (Hagen *et al*. 1998).

### *TUNEL assay*

The *in situ* cell death detection kit was purchased from Promega. The assays were carried out following the method described by Hagen *et al*. (1998). Briefly, approximately 30 microfilariae were transferred onto

https://doi.org/10.1017/S0031182001007545 Published online by Cambridge University Press

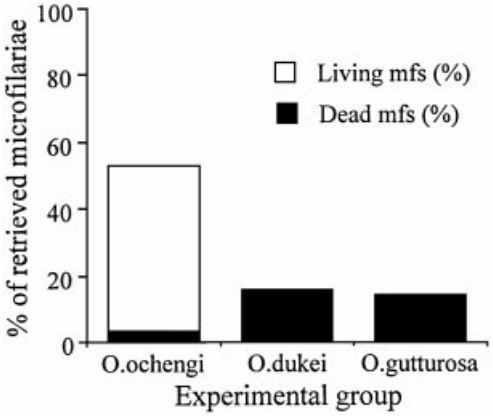


Fig. 1. *Simulium damnosum* s.l.: recovery rate of living and dead *Onchocerca ochengi* microfilariae (compatible) and *O. dukei* and *O. gutturosa* microfilariae (non-compatible species) 2 h after intrathoracic injection.

a 60 $\mu$l drop of double distilled water on a poly-L-lysine coated cavity slide using an eye-lash. After the slides were air-dried at 37 °C for 1 h, the changes of solutions were conducted under a dissection microscope as microfilariae are notoriously difficult to attach on glass. The scoring was done blind in that the level of fluorescence was evaluated on a scale from 0 to 4 without knowing the identity of the samples. The score was as follows: 0: no fluorescence, 1: single fluorescent cells, 2: less than half of cells fluorescent, 3: more than half of cell fluorescent, 4: all cells fluorescent.

### *Statistical analysis*

All data were tested for significant differences using the Mann–Whitney *U*-test. Broken microfilariae or worm fragments were excluded from the analysis of the TUNEL assays.

## RESULTS

### *Survival rates of different* Onchocerca *species*

About 50% of *O. ochengi* microfilariae survived the first 2 h p.i., whereas all of the *O. gutturosa* and *O. dukei* microfilariae were killed (Fig. 1), and their recovery rate was only around 15%. Interestingly nearly all of the dead *O. ochengi* microfilariae were already cleared at the time-point of dissection 2 h p.i. Dead *O. dukei* and *O. gutturosa* microfilariae were recovered in much higher numbers, as in these cases the blackfly's immune system had to cope with double the number of dead microfilariae during the clearance process. The differences in survival rates of the compatible species *O. ochengi* and the 2 other non-compatible species were very obvious. As none of the non-compatible microfilariae survived proper statistical analyses were redundant.

### *Survival rates in the presence of RGDS*

The increase of live microfilariae retrieved 24 h p.i. in the presence of the blocking agent RGDS (42 blackflies dissected) was highly significant when compared with the control group without any peptide ($P = 0{\cdot}0000$, 47 blackflies dissected) and the control group with the non-blocking peptide RGES ($P = 0{\cdot}0001$; blackflies dissected; Fig. 2). There was no significant difference between the RGES and the control group.

### *Induction of apoptosis in microfilariae in the presence of RGDS*

Due to the results of the survival trials described above only 2 groups, namely the RGDS and the RGES control group, were examined. After only 2 h exposure within the haemocoel the mean number of microfilariae retrieved from blackflies was already significantly higher in the RGDS group (10·1 mfs/fly) than in the RGES group (8·7 mfs/fly; $P = 0{\cdot}02$). At the same time the proportion of microfilariae showing apoptotic cells with fluorescence was significantly lower in the RGDS group than in the RGES group ($P = 0{\cdot}02$; Fig. 3). The differences of fluorescence were at the levels of 1 and 2 of the scoring scale.

## DISCUSSION

The developmental fate of a given parasite species in *S. damnosum* s.l. was determined within the first 2 h of microfilariae entering the haemocoel. As all of the non-compatible *Onchocerca* microfilariae had been killed during this initial exposure, it was concluded that the first 2 h upon entering the haemocoel are pivotal for the survival even for well-adapted parasites such as *O. ochengi*. The response did not completely discriminate between compatible and non-compatible species as only about half of the compatible *O. ochengi* microfilariae managed to survive the initial response. In this sense the rapid killing was in fact species specific. The recovered number of dead microfilariae in the case of *O. dukei* and *O. gutturosa* was much higher than for *O. ochengi*. This was certainly due to the fact that the system was 'saturated', having to clear 15 mfs instead of an average of 7 mfs in the case of *O. ochengi*. Unlike Lehmann *et al.* (1994), however, we did not conclude that the 'active factor' responsible for the microfilaricidal effects was a soluble one. The results from this study using the peptide RGDS to block cell adhesion suggested otherwise.

The presence of RGDS in the haemolymph led to a highly significant increased survival of *O. ochengi* mfs 24 h p.i when compared to the controls. This enhanced survival correlated with significantly reduced apoptosis levels 2 h p.i. in microfilariae co-

https://doi.org/10.1017/S0031182001007545 Published online by Cambridge University Press

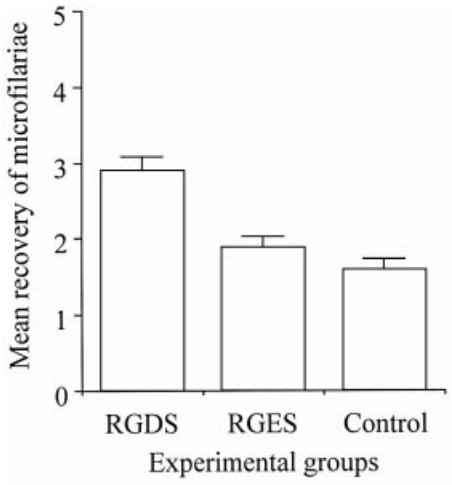


Fig. 2. *Simulium damnosum* s.l.: mean recovery rate of *Onchocerca ochengi* microfilariae 24 h after intrathoracic injection together with the peptide RGDS and 2 control groups, 1 without any peptide and 1 with the peptide RGES (RGDS > RGES: $P = 0{\cdot}0001$, RGDS > controls: $P = 0{\cdot}0000$).

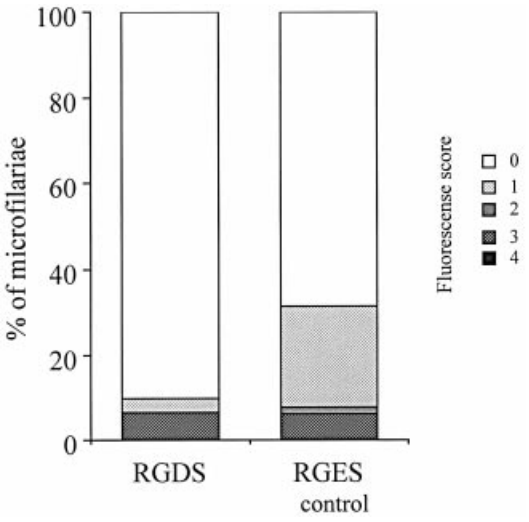


Fig. 3. *Onchocerca ochengi*: percentage of microfilariae showing different levels of apoptotic cells following 2 h *in vivo* exposure to the haemolymph of *Simulium damnosum* s.l. in the presence of the peptide RGDS or RGES as a control for specificity. Level of fluorescence in microfilariae due to apoptotic cells detected with a cell death detection assay (TUNEL) and scored from 0 = no cell to 4 = all cells fluorescent.

injected with RGDS group when compared to microfilariae co-injected with the peptide RGES as a control. Interestingly, in this experiment, where the exposure of microfilariae to blackfly haemolymph was only 2 h, the recovery rate of *O. ochengi* parasites was again significantly higher in the RGDS group than in the control RGES group. The results from this present study indicate that the rapid killing of *Onchocerca* microfilariae involved an integrin-like RGD-dependent cell adhesion mechanism. This would suggest that cellular components of the haemolymph were in fact taking part in the early response of blackflies to *Onchocerca* infection.

As these trials could have only been carried out *in vivo* it was not possible to directly observe the haemocyte-type involved during this process. However, during most of the 2 h of exposure, microfilariae are located in the haemolymph, with haemocytes representing the most likely cell type to be encountered by the parasite during their migration to the thoracic flight muscle. In most other vector insects this contact would lead to encapsulation by haemocytes but not in blackflies (Vernick, 1997). At least 4 blood cell types have been identified in *S. vittatum*, amongst them plasmatocytes and granular cells (Luckhart, Cupp & Cupp, 1992). Preliminary data with *S. damnosum* showed that the proportion of plasmatocytes present in the haemolymph of *S. damnosum* is highly significantly increased after 24 h p.i. as compared to granular cells (Hagen *et al.* unpublished observations). This observation was in line with findings by Cupp *et al.* (1997) who demonstrated that the number of plasmatocytes of *S. vittatum* increased during the course of an *Onchocerca* infection with a peak around 24 h p.i.

Our findings are very similar to observations made by Pech & Strand (1995). They demonstrated that RGDS-coated Sepharose beads were rapidly encapsulated (*in vitro* and *in vivo*) by plasmatocytes from the moth *Pseudopleusia includens* whereas granular cells were not. While both of these most important cell types are able to adhere to surfaces only plasmatocyte adhesion seemed to be specifically RGD-dependent. Interestingly the RGDS interfered with spreading of plasmatocytes at far lower concentrations (2 mM) than observed for granular cells (5 mM). RGES did have a slight effect on adhesion on plasmatocytes at 2 mM but none on granular cells. This compares well with our findings on the effects of soluble RGDS on the survival of microfilariae in their natural vector. Other studies have suggested that cell adhesion in an RGD-dependent manner is important for cellular immune response of invertebrates (Johansson, 1999). Glass-slivers coated with the peptide GRGDS induced spreading of haemocytes of the crustacean *Pacifastacus leniusculus* (Johansson & Soderhall, 1989). Haemocytes of snail *Biomphalaria glabrata*, intermediate host of the human parasite *Schistosoma mansoni*, were significantly inhibited in their ability to adhere when treated with the peptide RGDS (Davids & Yoshino, 1998). Interestingly, the depression of spreading was more effective in the susceptible than in the refractory strain of *B. glabrata*. Evidently the RGD-binding, integrin-like receptors of haemocytes play a critical role in the host defence of invertebrates other than blackflies as well. Recently an intergrin beta-subunit was identified and cloned from haemocytes of *P. leniusculus* (Holmblad *et al.* 1997).

https://doi.org/10.1017/S0031182001007545 Published online by Cambridge University Press

Despite the existence of a common adhesive RGD motif, integrin ligands belong to many different families of proteins amongst which are collagens (Johansson, 1999). Collagens are part of the cuticle of *Onchocerca* filariae (Henkle-Dührsen, Liebau & Walter, 1993), and some of those collagens are important for the immune response in humans living in endemic areas for river blindness (Garate *et al*. 1996; Stewart *et al*. 1997). It is therefore feasible that haemocytes could adhere to the surface of intruding *Onchocerca* microfilariae.

The strong correlation between the RGD-dependent survival and apoptotic levels observed in this present study would strongly imply the existence of cytotoxic haemocytes. Cytotoxic effects, previously reported by other authors, were mediated by reactive oxygen species, nitric oxide and/or other humoral molecules such as antibacterial peptides (Nappi & Ottavianni, 2000). Possible cytotoxic effects on microfilariae during their *in vivo* exposure to blackfly haemolymph appeared to be mediated via serine proteases leading to apoptosis and could be reversed by the generic caspase-inhibitor boc-D. fmk, excluding potential necrotic mechanisms (Hagen *et al*. 1998).

The circumstantial evidence so far of the direct cytotoxic effects of haemocytes has to be confirmed by more direct observations *in vitro*. Future work will concentrate on the question whether or not blackfly haemocytes have Natural Killer (NK) cell-like effector functions (see Humphreys & Reinherz, 1994). Research will focus on identifying elicitor and self-histocompatibility receptors on the haemocytes surface. This area will be of major importance as NK cell-like effector mechanisms, unlike in other invertebrate taxa, such as earthworm (Cooper *et al*. 1999) have been not reported from insects so far (Nappi & Ottavianni, 2000). We are confident that blackflies are an important model for elucidating the role of innate immunity in the context of parasitic infections in invertebrates and vertebrates alike.

We would like to thank the Wellcome Trust for the financial support throughout this study. We are also indebted to Dr V. N. Tanya, chief of station, of the Institut de Recherches Agricole pour le Development (IRAD) in Ngaoundéré-Wakwa, North-Cameroon, as well as Dr Klaus Ersfeld of the University of Manchester and Professor J. Bradley of the University of Salford, UK.

## REFERENCES

BAIN, O., DENKE, A. M., AMEGEE, Y. & CHABAUD, A. G. (1977). Les onchocerques des bovins au Togo: les microfilaires et leurs distributions. *Annales de Université de Benin* **3**, 117–123.

BIANCO, A. E., HAM., P. J., EL SINNARY, K. & NELSON, G. S. (1980). Large-scale recovery of *Onchocerca* microfilariae from naturally infected cattle and horses. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **74**, 109–110.

BWANGAMOI, O. (1969). *Onchocerca ochengi* new species, an intradermal parasite of cattle in East Africa. *Bulletin of Epizootic Diseases in Africa* **17**, 321–335.

COOPER, E .L., COSSARIZZA, A., KAUSCHKE, E. & FRANCESCHI, C. (1999). Cell adhesion and the immune system: a case study using earthworms. *Microscopical Research Techiques* **44**, 237–253.

CUPP, M. S., CHEN, Y. & CUPP, E. W. (1997). Cellular hemolymph response of *Simulium vittatum* (Diptera: Simuliidea) to intrathoracic injection of *Onchocerca lienalis* (Filarioidea: Onchocercidae) microfilariae. *Journal of Medical Entomology* **34**, 56–63.

DENKE, A. M. & BAIN, O. (1978). Donnees sur les cycle d'*Onchocerca ochengi* chez *Simulium damnosum* s.l. au Togo. *Annales de Parasitologie Humaine et Comparée* **53**, 757–760.

DAVIDS, B. J. & YOSHINO, T. P. (1998). Integrin-like RGD-dependent binding mechanism involved in the spreading response of circulating molluscan phagocytes. *Developmental and Comparative Immunology* **22**, 39–53.

GARATE, T., CONRATHS, F. J., HARNETT, W., BUTTNER, D. W. & PARKHOUSE, R. M. (1996). Identification of *Onchocerca volvulus* collagen as an antigen mainly recognised by antibodies in chronic hyper-reactive onchodermatitis (sowda). *American Journal of Tropical Medicine and Hygiene* **54**, 490–497.

HAGEN, H. E., GRUNEWALD, J. & HAM, P. J. (1994). Induction of the pro-phenoloxidase-activating system of *Simulium* (Diptera: Simuliidae) following *Onchocerca* (Nematoda: Filarioidea) infection. *Tropical Medicine and Parasitology* **45**, 13–16.

HAGEN, H. E., KLÄGER, S. L., CHAN, V., SAKANARI, J. A., MCKERROW, J. H. & HAM, P. J. (1995 *a*). *Simulium damnosum* s.l.: identification of inducible serine proteases following an *Onchocerca* infection by differential display reverse transcription PCR. *Experimental Parasitology* **81**, 249–254.

HAGEN, H. E., KLÄGER, S. L. & HAM, P. J. (1995 *b*). A simple method for the purification of *Onchocerca ochengi* microfilariae from mixed *Onchocerca* infection in cattle. *Tropical Medicine and Parasitology* **46**, 201–202.

HAGEN, H. E., KLÄGER, S. L., MCKERROW, J. H. & HAM, P. J. (1997 *a*). *Simulium damnosum* s.l.: isolation and identification of prophenoloxidase following infection with *Onchocerca* spp. using targeted differential display. *Experimental Parasitology* **86**, 213–218.

HAGEN, H. E., KLÄGER, S. L., BARRAULT, D. V. & HAM, P. J. (1997 *b*). The effects of protease inhibitors and sugars on the survival and development of the parasite *Onchocerca ochengi* in its natural intermediate host *Simulium damnosum* s.l. *Tropical Medicine and International Health* **2**, 211–217.

HAGEN, H. E., KLÄGER, S. L. & WILLIAMS, G. T. (1998). Is apoptosis involved in mechanisms to eliminate *Onchocerca ochengi* during *Simulium damnosum* s.l. immune response? *Tropical Medicine and International Health* **3**, 945–950.

HAM, P. J. (1986). Acquired resistance to *Onchocerca lienalis* infections in *Simulium ornatum* Meigen and *Simulium lineatum* Meigen following passive transfer of haemolymph from previously infected simuliids (Diptera, Simuliidae). *Parasitology* **92**, 269–277.

HAM, P. J., HAGEN, H. E., BAXTER, A. J. & GRUNEWALD, J. (1995). Mechanisms of resistance to *Onchocerca* infections in blackflies. *Parasitology Today* **11**, 63–67.

HENKLE-DÜHRSEN, K., LIEBAU, E. & WALTER, R. D. (1993). The *Onchocerca volvulus* mRNAs for a hsp70, a collagen-like protein and a ribosomal protein possess a 5′ spliced leader sequence. *Tropical Medicine and Parasitology* **44**, 337–339.

HOLMBLAD, T., THORNQVIST, P.-O., SODERHALL, K. & JOHANSSON, M. W. (1997). Identification and cloning of an integrin beta subunit from hemocytes of the freshwater crayfish *Pacifastacus leniusculus*. *Journal of Experimental Zoology* **277**, 255–261.

HUMPHREYS, T. & REINHERZ, E. L. (1994). Invertebrate immune recognition, natural immunity and the evolution of positive selection. *Immunology Today* **15**, 316–320.

JOHANSSON, M. W. (1999). Cell adhesion molecules in invertebrate immunity. *Developmental and Comparative Immunology* **23**, 303–315.

JOHANSSON, M. W. & SODERHALL, K. (1989). A peptide containing the cell adhesion sequence RGD can mediate degranulation and cell adhesion of crayfish granular haemocytes *in vitro*. *Insect Biochemistry* **19**, 573–579.

LEHMANN, T., CUPP, M. S. & CUPP, E. W. (1994). *Onchocerca lienalis*: rapid clearing of microfilariae within the blackfly *Simulium vittatum*. *Experimental Parasitology* **78**, 183–193.

LUCKHART, S., CUPP, M. S. & CUPP, E. W. (1992). Morphological and functional classification of the hemocytes of adult female *Simulium vittatum* (Diptera: Simuliidae). *Journal of Medical Entomology* **29**, 457–466.

NAPPI, A. J. & OTTOVIANI, E. (2000). Cytotoxicity and cytotoxic molecules in invertebrates. *BioEssays* **22**, 469–480.

NELSON, G. S. (1962). Observation on the development of *Setaria labiatopapillosa* using new techniques for infecting *Aedes aegypti* with the nematode. *Journal of Helminthology* **36**, 281–296.

PECH, L. L. & STRAND, R. (1995). Encapsulation of foreign targets by hemocytes of the moth *Pseudopleusia includens* (Lepidoptera: Noctuidae) involves an RGD-dependent cell adhesion mechanism. *Journal of Insect Physiology* **41**, 481–488.

RICHMAN, A. & KAFATOS, F. C. (1995). Immunity to eukaryotic parasites in vector insects. *Current Opinion in Immunology* **8**, 14–19.

STEWART, G. R., ZHU, Y., PARREDES, W., TREE, TI., GUDERIAN, R. & BRADLEY, J. E. (1997). The novel cuticular collagen Ovcol-1 of *Onchocerca volvulus* is preferentially recognised by immunoglobulin G3 from putatively immune individuals. *Infection and Immunity* **65**, 164–170.

VERNICK, K. D. (1997). Mechanisms of immunity and refractoriness in insect vectors of eukaryotic parasites. In *Molecular Mechanisms of Immune Responses in Insects* (ed. Brey, P. T. & Hultmark, D.), pp. 261–309. Chapman and Hall, London.

WAHL, G., EKALE, D. & SCHMITZ, A. (1998). *Onchocerca ochengi*: assessment of the *Simulium* vectors in North Cameroon. *Parasitology* **116**, 327–336.

WENK, P. & RAYBOULD, J. N. (1972). Mating, blood feeding and oviposition of *Simulium damnosum* Theobald in the laboratory. *Bulletin of the World Health Organization* **47**, 627–634.

WORLD HEALTH ORGANIZATION (1995) Onchocerciasis and its control. *WHO Technical Reports Series* No. 852.