Effects of an *Onchocerca*-derived cysteine protease inhibitor on microfilariae in their simuliid vector

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SUMMARY

A recombinant cysteine protease inhibitor, onchocystatin of the parasitic nematode *Onchocerca volvulus*, was tested for its role in microfilarial development in the simuliid vector. Onchocystatin was found to be present in female adults and skin microfilariae of the bovine parasite *O. ochengi*, the closest relative of *O. volvulus*. In addition the inhibitor could be detected as an excretory–secretory (E–S) product of the microfilariae. Co-injection of onchocystatin and the *O. ochengi* microfilariae into the surrogate vector *Simulium ornatum* s.l. significantly enhanced the recovery rates of the parasite within 24 h into the infection ($P > 0{\cdot}001$). The findings suggest a possible role of onchocystatin in the evasion by the parasite of the immune response of its vector.

Key words: filariasis, insect immunity, development, onchocystatin.

INTRODUCTION

The cysteine protease inhibitor onchocystatin was first discovered in an attempt to identify *Onchocerca*-specific antigens. When a λgt11 expression library of *O. volvulus* was screened with either human or chimpanzee infection sera several clones of the respective gene were recovered. One of these clones Ov7 was described in detail by Lustigman *et al*. (1992). Another clone, Ov10 was used as an antigen for serodiagnosis of human onchocerciasis (Bradley *et al*. 1993). However, the biological function for the parasite is not well established. Onchocystatin, with a molecular mass of 17000, is present in the hypodermis and the basal layer of the cuticle of L3, moulting L3, L4 and female adult worms, and in the egg-shell of developing larvae (Lustigman *et al*. 1992). No expression was observed in skin microfilariae at the time. It was suggested that the inhibitor was controlling the cysteine proteases which are vital for the moulting of the parasite larvae (Lustigman *et al*. 1996). Onchocystatin is highly immunogenic during infection (Bradley & Unnasch, 1996). Hartmann *et al*. (1997) showed, that a homologue of Ov10 from another filarial parasite, *Acanthocheilonema vitae*, is expressed in all developmental stages of the worm. It induces immunosuppression in the vertebrate host, suggesting that the released inhibitor could modulate the natural host–parasite relationship.

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This study was aimed to address the question of whether or not onchocystatin might facilitate early development of microfilariae in its insect host. We used *O. ochengi*, a bovine parasite transmitted by the same vector species as *O. volvulus* (Denke & Bain, 1978) which has proved a useful model in studying the transmission (Hagen, Grunewald & Ham, 1994) and immunobiology of human onchocerciasis (Trees *et al*. 1992). Previous studies have shown that by blocking cysteine proteases in the blackfly haemocoel with a respective inhibitor, the recovery rates of *O. ochengi* developing larvae increased (Hagen *et al*. 1997*a*). Initially it was necessary to investigate whether onchocystatin is expressed in *O. ochengi* and, more importantly, if it is expressed and released by the mature, skin-dwelling microfilariae.

MATERIALS AND METHODS

Source of blackfly and parasite material

Female *S. ornatum* s.l. were collected as larvae and pupae on trailing vegetation from a breeding site near Briggle Back/Cumbria, England and kept in large aerated water tanks. The adult flies, which are good surrogate vectors for *O. ochengi* and *O. volvulus* (McCall, Townson & Trees, 1992), were used for the experiments within 2 days of their emergence. The microfilariae of *O. ochengi* were isolated from bovine skin samples collected at the abattoir in Ngaoundere/Cameroon. The microfilariae were isolated following the method of Bianco *et al*. (1980) and cryopreserved according to Ham *et al*. (1981).

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Recombinant onchocystatin

The recombinant protein was prepared as described before (Bradley *et al*. 1993). Onchocystatin Ov10 was expressed as a fusion protein with the *Escherichia coli* maltose binding protein (MBP) in the pMal- expression vector (Maina *et al*. 1989). The recombinant proteins MBP-Ov10 and MBP were dialysed against phosphate-buffered saline (PBS) prior to use in the experiments. The inhibitory activity of the recombinant protein was described in detail by Lustigman *et al*. (1992) and confirmed for our preparation with the protease assay of Hagen *et al*. (1997*b*), using the cysteine protease papain (Sigma P3125) compared with the inhibitor E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, Sigma E3132. Trypsin (Sigma T4665), a serine protease was used for testing the specificity of MBP-Ov10, compared to a serine protease inhibitor AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride, Sigma A8456).

Demonstration of onchocystatin in O. ochengi

Polyclonal antiserum against recombinant MBP-Ov10 was raised in BALB/c mice by injecting 2×100 μl of 100 μg/ml MBP-Ov10 in the MPL-TDM adjuvant system (Sigma M6536). Titre and specificity was tested by ELISA and the sera were collected after the third boost. Somatic antigen of *O. volvulus* and *O. ochengi* adult females, and of 100000 *O. ochengi* microfilariae was prepared according to the method of Maizels *et al*. (1991). These antigen preparations and the recombinant MBP-Ov10 and MBP were separated on a 12% tricine–SDS–polyacrylamide gel (Schägger & von Jagow, 1987), blotted onto nitrocellulose membrane and probed with the polyclonal mouse anti-MBP-Ov10 serum, normal mouse serum, rabbit anti-MBP serum (New England Biolabs, Beverly, MA), and normal rabbit serum. Peroxidase conjugated anti-mouse immunoglobulins or anti-rabbit IgG were used respectively for detection.

O. ochengi microfilariae were metabolically labelled with [^{35}S]methionine for immunoprecipitation using 60000 microfilariae each in 2 independent experiments. Cryopreserved microfilariae were transferred into RPMI 1640 (0·2 mg/ml gentamycin) and kept for 1 h at 37 °C. They were washed twice in methionine-free medium, transferred with 500 μl of medium into a well of a 4-well cell culture plate and starved for 4 h. Subsequently, 250 μl of medium were removed and 250 μl of medium containing [^{35}S]methionine were added to a final concentration of 5·3 MBq/ml. Supernatant and microfilariae were collected after an incubation period of 24 h at 37 °C and 5% CO_2. For each experiment the collected supernatant was divided into 2 equal portions and used for immunoprecipitation with the polyclonal mouse anti-MBP-Ov10 serum and pre-immune normal mouse serum as control. A microfilarial extract was produced by snapfreezing the parasites in PBS with 1% SDS and 2·5% Triton X–100 in liquid nitrogen and subsequent thawing at 37 °C (5 cycles). This preparation was set for 30 min on ice and then centrifuged at 12000 ***g*** for 15 min. Immunoprecipitations were conducted as described by Maizels *et al*. (1991) with the following alterations. Sepharose A beads were first covered with rabbit anti-mouse IgG, before the incubation with the antigen–antibody complex. For each experiment the samples were run on a 12% tricine–SDS–polyacrylamide gel, fixed and treated with an amplification solution according to the manufacturer's instructions (Amersham), dried under vacuum and exposed to film.

Infection and fly maintenance

The *O. ochengi* microfilariae were identified morphologically (Bain *et al*. 1971). Five *O. ochengi* microfilariae, which had been proved to be an ideal number to keep the injection volume low (Hagen *et al*. 1997*a*), were injected into blackflies through the pleural membrane at the base of the wing (Nelson, 1962). The infected flies were kept for 24 h in polystyrene pots placed in a propagator, and fed on 10% sugar solution supplemented with Nipagin (0·25% w/v), penicillin (200 U/ml) and streptomycin (200 μg/ml).

Co-injection experiments with onchocystatin

Co-injections were carried out as described previously (Hagen *et al*. 1997*a*). Flies were injected with either 1 μl of 45 μM MBP-Ov10 and 5 microfilariae (final volume 1·5 μl), 1 μl of 45 μM MBP and 5 microfilariae (final volume 1·5 μl) or 5 microfilariae in a volume of 1·5 μl RPMI 1640 cell culture medium. The 45 μM solutions of MBP-Ov10 and MBP were made up from stock solutions in RPMI 1640 medium. The estimated final concentration in the flies was 15 μM, respectively. The flies were dissected 24 h after infection and the number of larvae counted. Results were analysed with the Student's *t*-test to determine whether differences in the recovery rates of the experimental groups were significant at a confidence level of 95%. χ^2-test ($n \times m$ table) was used to compare the proportions of flies with different numbers of recovered microfilariae.

RESULTS

Demonstration of onchocystatin in O. ochengi

We used the polyclonal antiserum raised against the recombinant onchocystatin of *O. volvulus*, MBP-

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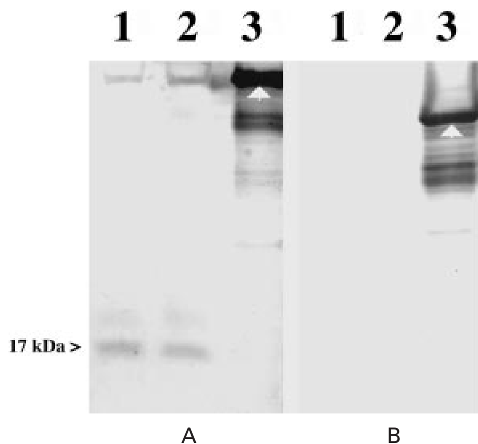

Fig. 1. Demonstration of onchocystatin by immunoblot analysis. Somatic worm extracts of adult females of *Onchocerca ochengi* (lane 1) and *O. volvulus* (lane 2), and of the recombinant onchocystatin, the fusion protein MBP-Ov10 (lane 3) were separated on a 12 % Tricine–SDS–polyacrylamide gel, blotted, and incubated with either polyclonal mouse antiserum directed against MBP-Ov10 (A) or rabbit antiserum directed against the carrier protein MBP only (B). The mouse anti-onchocystatin serum recognized the recombinant fusion protein of 57·7 kDa (white arrow) and the native 17 kDa protein in the female parasites. The rabbit anti-MBP serum recognized only the recombinant fusion protein.

Ov10, in a immunoblot analysis of female *O. volvulus* and *O. ochengi* (Fig. 1). A band of 17 kDa was recognized in the females of both species. Pre-immune mouse serum, rabbit anti-MBP and normal rabbit serum were used as controls to ensure the specificity of the band. A 17 kDa product was also recognized in the antigen produced from 100000 *O. ochengi* microfilariae, however, these blots were too faint for documentation. The 2 immunoprecipitation experiments with the polyclonal anti-MBP-Ov10 sera and the extracts of 60000 radioactive metabolically labelled microfilariae each and their culture supernatants showed the presence of a 17 kDa product in the somatic extract of microfilariae and their E–S products (Fig. 2).

Activity of the fusion protein MBP-OV10

The recombinant onchocystatin proved to have specific inhibitory activity on the protease assay plates. The activity of 100 μg/ml papain was reduced by 80 % in the presence of 4 μM MBP-Ov10, whereas 4 μM MBP showed no inhibition. The inhibitor E-64 (20 μM) inhibited 99 % of the papain activity. MBP-Ov10 and MBP did not inhibit the activity of the serine protease trypsin, whereas 4 mM AEBSF reduced its activity by 90 %.

Co-injection of onchocystatin and microfilariae

The co-injection experiments were repeated 3 times using different batches of blackflies and microfilariae

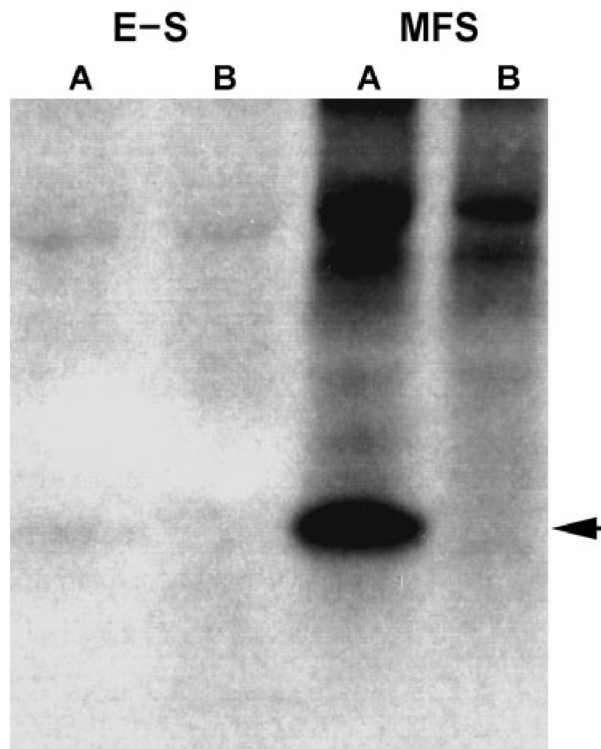

Fig. 2. Detection of onchocystatin in *Onchocerca ochengi* microfilariae and their excretory–secretory (E–S) products by immunoprecipitation. Somatic extract of metabolically labelled microfilariae (MFS) and their E–S products were precipitated with a polyclonal mouse antiserum directed against the recombinant fusion protein MBP-Ov10 (A) or pre-immune mouse serum (B) as control. In both, the microfilarial extract and the E–S products, the native 17 kDa onchocystatin was precipitated by the anti-MBP-Ov10 serum, as seen on the radiograph, and not by the pre-immune serum, whereas all the other products were precipitated by the pre-immune and immune sera.

Table 1. Proportion of *Simulium ornatum* s.l. found with 0, 1, 2, 3, 4 or 5 microfilariae 24 h after injection of 5 *Onchocerca ochengi* microfilariae together with onchocystatin (inhibitor group) or without inhibitor (controls A and B)

Percentage of flies found with:	Control group A (no protein)	Control group B (recombinant MBP)	Inhibitor group (recombinant MBP-Ov10)
0 mfs	14·1	12·2	6·8
1 mfs	33·3	34·1	21·9
2 mfs	43·6	42·7	39·7
3 mfs	6·4	7·3	23·3
4 mfs	1·3	3·7	6·8
5 mfs	1·3	0	1·4
Number of flies dissected	78	82	73

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Table 2. Mean recovery rates of *Onchocerca ochengi* microfilariae 24 h after injection of 5 microfilariae into *Simulium ornatum* s.l. with onchocystatin (inhibitor group) or without inhibitor (controls A and B); *P* values for Student's *t*-test comparing control group A and inhibitor group; number of dissected blackflies in parentheses, and standard errors

Trial	Control group A (no protein)	Control group B (recombinant MBP)	Inhibitor group (recombinant MBP-Ov10)	*P* values
1	1·467 (30) 0·142	1·700 (30) 0·180	2·130 (23) 0·211	$< 0·01$
2	1·286 (28) 0·177	1·300 (30) 0·153	1·688 (32) 0·193	$> 0·05$
3	1·900 (20) 0·250	1·727 (22) 0·199	2·611 (18) 0·200	$< 0·02$
Summary	1·526 (78) 0·108	1·561 (82) 0·103	2·055 (73) 0·125	$< 0·001$

for each injection trial. Each trial consisted of 3 experimental blackfly groups: Control group A, injected with 5 *O. ochengi* microfilariae; Control group B, injected with 5 *O. ochengi* microfilariae and the recombinant carrier protein MBP; Inhibitor group, injected with 5 *O. ochengi* microfilariae and the recombinant onchocystatin MBP-Ov10. Dissection of the blackflies was carried out 24 h later in RPMI 1640 cell culture medium and the recovered microfilariae counted. In total, 233 blackflies were dissected. The proportion of flies found with none, 1, 2, or 3–5 microfilariae 24 h into infection did not differ between the 2 control groups (Table 1; $\chi^2 = 0·29$, D.F. $= 3$, $P = 0·963$). The co-injection of the cysteine inhibitor resulted in a significant shift towards flies found with 3 and more microfilariae from 9 and 11% respectively in the 2 control groups to 31·5% in the inhibitor group ($\chi^2 = 13·41$, D.F. $= 3$, $P < 0·0033$ for inhibitor compared to control group A; $\chi^2 = 11·14$, D.F. $= 3$, $P < 0·0011$ for inhibitor compared to Control group B). The number of flies dissected per group and the mean recovery rate per group are listed in Table 2. In summary, the mean recovery rate of microfilariae in Control group A of 1·513 and in Control group B of 1·561 did not differ significantly, whereas the mean recovery rate of 2·055 of the inhibitor group was significantly higher ($P < 0·001$) than the 2 controls.

DISCUSSION

This study clearly shows that onchocystatin is present in *O. ochengi*. More importantly it could be demonstrated in 2 independent metabolic labelling experiments for the first time that *Onchocerca* skin microfilariae express and secrete this protein. The lack of sufficiently high numbers of *O. volvulus* microfilariae in previous studies might explain why it has not been observed before. The fact that onchocystatin is released by microfilariae into the surrounding environment of host tissues increases its ability to influence the host–parasite relationship.

In this study we co-injected recombinant onchocystatin together with microfilariae into the blackfly host. The presence of the inhibitor resulted in a 3-fold increase in the proportion of blackflies found with 3 or more microfilariae. The mean recovery of microfilariae injected together with the inhibitor was consistently higher in all 3 trials than in the respective 2 control groups without inhibitor. The results were significant for trials 1 and 3, and for the pooled data. The overall low recovery rates in all 3 groups of trial 2 were most probably due to the reduced viability of the batch of cryopreserved microfilariae used. The results confirm the former observation (Hagen *et al.* 1997*a*) that the cysteine protease inhibitor E-64 enhanced the development rate of microfilariae to infective larvae.

It is well established that *Onchocerca* microfilariae have to mature *ex uteri* to become infective for the insect vector (Townson & Tagboto, 1996). Skin microfilariae of *O. cervipedes*, *O. cervicalis* and *O. lienalis* have a modified protease expression, which might facilitate migration through the skin (Lackey *et al.* 1989) and in *O. volvulus* skin microfilariae collagenase activity was demonstrated (Petralanda, Yarzabahal & Piessons, 1986). These microfilarial enzymes might also be important for the penetration of gut and flight muscle of insect vectors (Perrone & Spielmann, 1986). The skin microfilariae's own cysteine protease inhibitor would be a further example of a parasite molecule important for the successful establishment within the insect vector.

Research on the innate immune system of blackflies has revealed that it plays an important part in controlling the development of the parasite and might be involved in determining the specificity and vector capacity of a given *S. damnosum* s.l. cyto-

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species or population (Ham *et al*. 1995). In spite of lacking the ability to encapsulate and melanize intruding microfilariae, blackflies are capable of killing and clearing the *Onchocerca* parasite in a rapid (Lehmann, Cupp & Cupp, 1994) and species-specific manner (Hagen, unpublished observation). The presence of cysteine proteases in blackflies has not yet been investigated. However, a cathepsin L-like cysteine protease is expressed in *Drosophila melanogaster* haemocytes where it plays probably an important role in insect immunity (Tryselius & Hultmark, 1997). The fact that onchocystatin is released by microfilariae and the co-injection of microfilariae with onchocystatin significantly reduced the clearing of the parasites in the simuliid vector suggests that it is able to interfere in the insect immune response. It is postulated that the ability of onchocystatin to facilitate establishment of the microfilariae in the insect is based on the blocking of an immuno-relevant cystatin protease of the blackfly.

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