Expression of an *Onchocerca volvulus* Ov33 homolog in *Dirofilaria immitis*: potential in immunodiagnosis of heartworm infection

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**SUMMARY**

In this study, the expression of an *Onchocerca volvulus* Ov33 homolog was demonstrated in *Dirofilaria immitis*. Rabbit antiserum raised against a recombinant fusion protein of *O. volvulus*, MBP/OvD 5B (Ov33), was found to react with a 31–33 kDa glycoprotein (DiT33) of adult worms of *D. immitis*. An antibody response to MBP/OvD 5B was observed in dogs, as early as 11 weeks post infection with infective larvae of *D. immitis*, and in dogs with occult infection. Cats both experimentally and naturally infected with *D. immitis* also reacted strongly with the recombinant antigen. In contrast, sera from dogs receiving chemically-abbreviated infection or from animals harbouring a variety of other helminths failed to react. These data suggest that antibody responses generated by DiT33 may have potential in immunodiagnosis of heartworm infection in cats and dogs.

**Keywords** D. immitis, O. volvulus, Ov33, antibody, diagnosis, lectin, jacalin

**INTRODUCTION**

*Dirofilaria immitis* is a common filarial nematode of dogs, cats and wild canids and possesses many of the features characteristic of filarial parasites of humans. Due to these similarities, *D. immitis* is considered a valuable model system for the major human filarial parasites *Brugia malayi*, *Wuchereria bancrofti* and *Onchocerca volvulus*. Diagnosis of heartworm (*D. immitis*) infection is currently performed by examining blood for the presence of microfilariae or by using commercially available tests which detect circulating antigen. Both methods rely on the presence of fecund female worms which are found approximately 6–5 months post infection (American Heartworm Society 1992). A procedure for detecting pre-patent infection would be desirable in the management of heartworm infection.

An immunodominant antigen of *Onchocerca volvulus*, Ov33-3 (Ov33), has been described which has potential in immunodiagnosis of onchocerciasis (Lucius et al. 1988a). This antigen has been cloned (Lucius et al. 1988b) and the antibody response to the recombinant antigen characterized (Lucius et al. 1992). Almost identical clones, Oc 3.6 (Chandrashekar et al. 1991) and OvD 5B (Dissanayake et al. 1993, C.N., PhD thesis, University of Yaounde, Cameroon), have been isolated independently by other groups using antibodies. Despite the specific seroreactivity observed in humans, homolog(s) of this antigen exist in other filarial parasites. The rodent filarial parasite *Acanthocheilonema viteae* possesses a homolog (Av33), which was identified using an Ov33 cDNA probe. Av33 and Ov33 were shown to possess significant homology to an aspartyl protease inhibitor of *Ascaris suum* (Willenbucher, Hofle & Lucius 1993).

In this study we demonstrate the presence of a 31–33 kDa glycoprotein in *D. immitis* (DiT33) which repre-
sents another homolog of Ov33. Using a recombinant fusion protein of *O. volvulus*, OvD 5B, we show that antibody responses to DiT33 may have significant potential in immunodiagnosis of heartworm infection.

**MATERIALS AND METHODS**

**Parasites**

Adult worms, microfilariae and mosquitoes (*Aedes aegypti*) containing infective larvae of *D. immitis* were purchased from TRS Laboratories Inc., Athens, GA, USA. Infective larvae were collected from cold anaesthetized mosquitoes using a Baermann apparatus. Female worms of *O. volvulus* were dissected from nodules kindly provided by Prof. P.J.Ham, University of Keele, Staffordshire, UK.

**Parasite extracts**

Ten adult male worms of *D. immitis* were pulverized on dry ice and homogenized in 10 ml phosphate buffered saline (PBS) containing a cocktail of protease inhibitors (Boehringer Mannheim, Indianapolis, IN, USA) comprising antipain dihydrochloride 50 µg/ml, APMSF 10 µg/ml, aprotinin 1 µg/ml, bestatin 4 µg/ml, chymostatin 10 µg/ml, E-64 5 µg/ml, EDTA 1 mM, leupeptin 0.5 µg/ml, pepstatin 0.7 µg/ml and phosphoramidon 10 µg/ml. The homogenate was centrifuged (100 000 g, for 1 h) to obtain a soluble fraction (PBS extract) and stored at −70°C.

Excretory/secretory (E/S) antigen of *D. immitis* was obtained from adult worms essentially as described (Poole et al. 1992). Worms were incubated in RPMI 1640 medium, 1% (wt/vol) glucose, penicillin 100 µ/ml, streptomycin 100 µg/ml, and amphotericin 25 µg/ml (Gibco/BRL, Gaithersburg, MD, USA), at 37°C and 5% CO2. Spent media was pooled, filtered (0.22 µm) and concentrated using an ultrafiltration stirred cell containing an Mr 10 000-cutoff Diaflo membrane (Amicon, Beverly, MA, USA).

An SDS extract of adult female worms of *O. volvulus* was prepared by homogenizing parasites in 1% SDS. The homogenate was processed as above.

**Production of MBP/OvD 5B**

OvD 5B was cloned as described (Dissanayake et al. 1993, C.N. PhD thesis, University of Yaounde, Cameroon). The original lambda gt11 phage was isolated from an *O. volvulus* adult worm cDNA library (Donelson et al. 1988) by screening for *O. volvulus* specific antigens and then subcloned for over-expression in the Protein Fusion and Purification System (New England Biolabs, Beverly, MA, USA). Recombinant MBP/OvD 5B is a fusion protein with the maltose binding protein (MBP) and an allele of Ov33. Partial DNA sequence of the 5' and 3' ends of OvD 5B indicates that it contains the entire region of Ov33-3 described by Lucius et al. 1988b, plus seven additional amino acids (Asn-Leu-Gly-Ile-Ile-Pro-Ser) at the N-terminus.

MBP/OvD 5B was induced and purified on amylose resin as described by the manufacturer (NEB) with the following exceptions. Cultures were grown to mid log phase and then induced overnight at 20°C. After elution from the amylose resin, the fusion protein was further purified by passage over a FPLC MonoQ column (Pharmacia, Piscataway, NJ, USA).

**Sera**

Dog and cat sera were purchased from TRS Laboratories. For species-specificity studies, serum samples were collected from dogs harbouring one or more of the following common parasites: *Toxocara canis, Toxascaris leonina, Ancylostoma caninum, Dipetalonema reconditum, Taenia spp.*, or from cats with uncharacterized, intestinal helminth infection. Animals experimentally infected with *D. immitis* were known to lack intestinal helminths or infection with *D. reconditum* (dogs). Rabbit anti-MBP/OvD 5B antibodies were generated in New Zealand White rabbits following immunization with 100 µg MBP/OvD 5B in Freund's Complete Adjuvant (Sigma St. Louis, MO, USA) given intramuscularly. The animals received a similar injection in Freund's Incomplete Adjuvant after two weeks (intramuscular) and six weeks (subcutaneous).

**Affinity purification of native antigens from *O. volvulus* and *D. immitis* using rabbit anti-MBP/OvD 5B**

Rabbit anti-MBP/OvD 5B antibodies were bound to protein A-agarose beads (Sigma). 100 µl of beads was added to 1 ml of *D. immitis* male worm PBS extract (1·6 mg/ml), or 1 ml of a SDS extract of *O. volvulus* (diluted 1:10 with PBS containing 1% Triton X-100). After incubation for 1 h at room temperature the beads were washed three times with PBS 0·1% Triton X-100 (PBS-T). Immunopurified antigens were eluted with 200 µl of 0·1 M glycine buffer pH 2·5, and the pH neutralized with 1 M Tris pH 8·0.

**Immunoblotting**

MBP/OvD 5B (2 µg), intact infective larvae (180/lane),
microfilariae (5000/lane), adult worm PBS extract (6.4 μg/lane) or E/S (3.2 μg/lane) or the immunopurified material (30 μg) were boiled for 5 min in SDS-PAGE sample cocktail (containing 3.2 M urea, 1% SDS and 5% 2-mercaptoethanol final concentration). Samples were microfuged for 5 min and the supernatants were electro-phoresed on 10–20% SDS-PAGE mini-gels (Diiachi, Tokyo, Japan) and transferred to nitrocellulose. Nitrocellulose membranes were stained with Ponceau material (30 pg) were boiled for 5 min in SDS-PAGE (Rockford, IL, USA). Biotinylated Lycopersicon esculentum (LEA) and Vicia villosa isolectin B4 (VvB4) were purchased from Sigma. After electrophoresis and transfer, nitrocellulose membranes containing O. volvulus or D. immitis antigens affinity purified with rabbit anti-MBP/OvD 5B were blocked with 1% BSA in PBS (PBS-BSA) for 1 h at room temperature. Each strip was then incubated with 3 ml of a biotinylated lectin (20 μg/ml in PBS-BSA), washed with PBS-T, and developed using the avidin-biotin-peroxidase system described above.

RESULTS

Expression of an Ov33 homolog by various stages of D. immitis

Polyclonal rabbit sera generated against MBP/OvD 5B was found to react with a doublet of 31 and 33 kDa present in PBS extracts of adult male worms, male E/S and microfilarial extracts of D. immitis (Figure 1). In addition, a minor component of approximately 21 kDa was also detected in both adult PBS extract and E/S preparations. Neither of these antigens were recognized by rabbit antibodies raised against MBP (data not shown). Infective larvae did not possess any molecule reactive with rabbit anti-MBP/OvD 5B sera.

Antibody response of dogs to MBP/OvD 5B during the course of infection with D. immitis

To determine if the D. immitis homolog of Ov33 was antigenic, pooled sera from four dogs experimentally infected with D. immitis were examined for reactivity with MBP/OvD 5B on weeks 0, 5, 11, 15, 21 and 27 post-infection (p.i.). An IgG response was initially detected in immunoblots 11 weeks p.i. which subsequently increased (Figure 2). When the sera were examined individually by ELISA, a transient decrease in response was observed in three dogs around 15 weeks p.i. (data not shown). The responses observed were directed against OvD 5B since these dogs did not possess anti-MBP antibodies (data not shown). At week 27, when the animals were necropsied, comparable numbers (32–41) of adult worms were recovered from the hearts of all four dogs. Patency normally begins at approximately 6.5 months p.i. with

Enzyme-linked immunosorbant assay (ELISA)

Wells of microtitre plates (Dynatech Laboratories, Chantilly, VA, USA) were coated overnight at 4°C with an optimum concentration of MBP fusion protein (5 ng/well) in 0.05 M carbonate buffer, pH 9.6. Following two washes in 0.05% (v/v) Tween 20 (PBS-Tween), plates were blocked for 30 min with 2% milk in PBS-Tween.

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Lectin blotting

The extent of glycosylation of Ov-33 and DiT-33 was determined by lectin blot analysis. Biotinylated Concanavalin A (Con-A), Erythrina cristagalli lectin (ECL), Artocarpus integrifolia (jacalin), peanut agglutinin (PBA), Phaseolus vulgaris erythroxagglutinin (PHA-E), Ricinus communis agglutinin I (RCA120, soybean agglutinin (SBA), Sophora japonica agglutinin (SJA), and wheat germ agglutinin (WGA) were purchased from Pierce (Rockford, IL, USA). Biotinylated Lycopersicon esculentum (LEA) and Vicia villosa isolectin B4 (VvB4) were purchased from Sigma. After electrophoresis and transfer, nitrocellulose membranes containing O. volvulus or D. immitis antigens affinity purified with rabbit anti-MBP/OvD 5B were blocked with 1% BSA in PBS (PBS-BSA) for 1 h at room temperature. Each strip was then incubated with 3 ml of a biotinylated lectin (20 μg/ml in PBS-BSA), washed with PBS-T, and developed using the avidin-biotin-peroxidase system described above.

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Figure 1 Reactivity of rabbit anti-MBP/OvD 5B antisera with various stages of *D. immitis*. Adult male PBS extract (lane 1), adult male E/S antigen (lane 2), infective larvae (lane 3) and microfilariae (lane 4) were examined in immunoblots. Arrows indicate 31–33 and 21 kDa antigens.

*D. immitis*. At necropsy, two of the four animals in this study were patent.

**Antibody response of dogs with occult heartworm infection**

Occult infection, in which dogs harbour a substantial number of adult worms without circulating microfilariae, occurs in a significant percentage of animals and is undetectable by current parasitological tests. The reactivity to MBP/OvD 5B in a group of 15 dogs with occult infection was analysed. Antibodies were detected in 13 animals.

**Antibody response of dogs following heartworm chemoprophylaxis**

The antibody response to MBP/OvD 5B was examined in 16 dogs which received an infection comprising 50 infective larvae of *D. immitis*. Four weeks after infection all animals received 50 μg/kg ivermectin and serum was collected four months p.i. Six months p.i., animals were necropsied and no adult worms were recovered. Of the 16 sera tested, only one was weakly reactive with MBP/OvD 5B.

Species-specific antibody response of dogs to MBP/OvD 5B

To determine if the responses observed in dogs were species-specific, six serum samples collected from animals harbouring one or more of the following common parasites of dogs were assayed for reactivity with MBP/OvD 5B: *Toxocara canis, Toxascaris leonina, Ancylostoma caninum, Dipetalonema reconditum*, and *Taenia* spp. Sera collected from uninfected (negative) or *D. immitis* infected (positive) dogs were included as controls. Only the positive control sera reacted strongly with the recombinant antigen (Figure 3). A total of 43 additional sera collected from dogs with helminth infections other than *D. immitis* were analysed in the same manner and none was positive (data not shown).

Antibody response of cats infected with *D. immitis*

Infection in cats is typified by the presence of few adult worms and a transient or absent microfilaraemia. In this study, two cats which were experimentally infected with 100 L3 larvae of *D. immitis*, possessed only two and nine adult worms at necropsy and no microfilaraemia at
weeks 24–28 p.i. These animals displayed a strong IgG response to MBP/OvD 5B (Table 1). Responses were detected earlier following transplant of 5–8 adult male and female worms. Comparable responses were obtained when sera were analysed at 4–40 weeks post-transplantation (Table 1).

Sera from 13 cats obtained from animal shelters in the southern United States were examined for reactivity to MBP/OvD 5B. A significant response was only observed in the two cats found to possess adult worms of *D. immitis*. The remaining 11 animals which possessed either intestinal helminths or no evidence of any helminthiasis did not react with the recombinant antigen (data not shown).

**Carbohydrate analysis of Ov33 and DiT33 native antigens**

The native antigens of adult *O. volvulus* and *D. immitis* recognized by rabbit anti-MBP/OvD 5B sera were affinity purified and characterized further by lectin blot analysis. Several lectins were selected based on their differential carbohydrate-binding specificities: Con-A (mannose/glucose), jacalin (galactosylo(β-1,3)N-acetylgalactosamine), ECL (N-Acetyllactosamine), LEA (N-acetylglucosamine oligomers), PNA (galactosyl end groups), PHA-E (complex type sugars), SBA and RCA (N-acetylgalactosamine/galactose), SJ2 (β-D-N-acetylgalactosamine), VvB4 (N-acetyl-D-galactosamine) and WGA (N-acetylgalactosamine). Of all the lectins tested, only jacalin was found to react with the 31–33 kDa molecules of both *O. volvulus* and *D. immitis* (Figure 4). In reciprocal experiments in which the 31–33 kDa molecules were affinity purified from *D. immitis* adult extract using jacalin, a strong reactivity with the rabbit anti-MBP 5B/OvD sera was observed (data not shown).

**DISCUSSION**

The presence of a homolog of the *O. volvulus* antigen Ov33 in *D. immitis* is suggested in these studies. Using rabbit anti-MBP/OvD 5B antisera in immunoblots, a doublet of 31 and 33 kDa was observed in adult male, adult excretory/secretory components and microfilarial extracts of *D. immitis*. An additional band of 21 kDa present in adult worm material was recognized by anti-MBP/OvD 5B antibodies. These antibodies did not react with any antigens from infective larvae. Previous results indicate that *O. volvulus* parasites express the Ov33 molecule in a similar manner (Lucius *et al.* 1988a).

Sera from chimpanzees experimentally infected with *O. volvulus*, were examined for reactivity with recombinant Ov33 and antibodies were detected around patency (Lucius *et al.* 1992). In the present study, we show that sera collected from dogs experimentally infected with *D. immitis*, including animals with occult infection, were strongly reactive with the MBP/OvD 5B fusion polypeptide. In contrast to the chimpanzee data, the response to

**Figure 3** Reactivity of sera from dogs harbouring various helminth infections. The IgG response to MBP/OvD 5B of dogs harbouring *D. immitis* (□), *Dipetalonema reconditum* (△) or intestinal worms (○) was examined by ELISA.

**Table 1** IgG response of *D. immitis* infected cats to MBP/OvD 5B in ELISA

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<th>Months after L3 infection&lt;sup&gt;2&lt;/sup&gt;</th>
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<sup>1</sup> Each value (OD 495 nm) represents the response of a single cat.

<sup>2</sup> Animals infected with 100 L3 larvae of *D. immitis*.

<sup>3</sup> Animals infected with 5–8 worms (both sexes) by injection in the jugular vein.
the Ov33 homolog in D. immitis as assayed with MBP/OvD 5B, was initially detected 11 weeks p.i., coinciding with the L4/L5 moult in dogs. A relatively large number of dogs infected with D. immitis develop occult infections which have no circulating microfilariae. These animals obviously present a challenge for diagnosis yet 87% of occult animals tested in this study displayed a significant response to MBP/OvD 5B.

Heartworm infection is largely controlled today by chemoprophylaxis. The infrequent occurrence of parasites in treated dogs is a result of the high efficacy of currently available drugs. However, brief lapses in drug administration or insufficient dosage may result in a mature infection with associated pathology. In the present study, we demonstrated that animals treated with anthelminthic one month p.i. did not respond to MBP/OvD 5B. This suggests that we may be able to distinguish dogs with adult worm infection from animals which have only been exposed to early larval forms.

Results of Southern blots indicated that homologs of Ov33 exist in B. malayi and D. immitis (Lucius et al. 1988b). The gene has recently been cloned in B. malayi (Bm33) (Dissanayake et al. 1993) and Acanthocheilonema viteae (Av33) (Willenbucher et al. 1993). Interestingly, despite the sharing of antigenic determinants by members of this family of proteins, Ov33 has been shown to have potential in diagnosis of onchocerciasis. High levels of specificity can be achieved based on the detection of IgG4 responses to Ov33 (Lucius et al. 1992). We have demonstrated significant antigenic cross-reactivity between the O. volvulus and D. immitis molecules. This relationship apparently does not extend to other parasites of cats and dogs, since a large number of sera collected from animals harbouring various other helminth infections did not react with the O. volvulus fusion protein.

The ability to necropsy animals infected with D. immitis offers a unique opportunity to correlate antibody responses and worm burden. In cats which were experimentally infected with infective larvae or received transplants of adult worms, we were able to show a significant response to MBP/OvD 5B. We were also able to detect responses in cats naturally infected with D. immitis, despite the fact that these animals typically have low numbers of adult worms and few or no microfilariae.

In addition to the antigenic crossreactivity between Ov33 and DiT33 observed at the protein level, the similarities extend to their post-translational modifications. Jacalin, which is specific for the O-linked T antigen (a tumour cell marker) (Sastry et al. 1986), was the only lectin found to bind DiT33 or Ov33. We have previously shown that jacalin binds to a large number of molecules in various stages of D. immitis (Meija & Carlow 1994). Further characterization of DiT33 using O-glycanase revealed that the upper band of the doublet (33 kDa) was fully resistant, while the lower band (31 kDa) contained both resistant and susceptible moieties (data not shown). Lectin blot analysis of both Ov33 and DiT33 suggested an absence of N-linked glycans. This is consistent with our observation that PNGase F does not affect DiT33 (data not shown).
It has been shown that Ov33 and Av33 possess significant homology with an aspartyl protease inhibitor of *Ascaris suum*, and postulated that these molecules inhibit proteases released by inflammatory cells (Willenbucher, Hofle & Lucius 1993).

Our overall findings reveal a close relationship between the *O. volvulus* Ov33 and *D. immitis* DiT33 molecules. In addition, we have shown that responses to Ov33/DiT33 can be detected in prepatent or occult cats and dogs. Therefore these antigens may be useful in diagnosis of heartworm infection and in the assessment of experimental *D. immitis* infections in drug and vaccine trials.

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