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SHORT COMMUNICATION

Immunolocalization of Disorganized Muscle Protein-1 in Different Life Stages of Human Lymphatic Filariid, *Brugia malayi*

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Abstract

Purpose We recently identified disorganized muscle protein-1 of *Brugia malayi* (DIM-1bm) as a vaccine candidate for human lymphatic filariasis. The present study was aimed at investigating the localization of DIM-1bm in the life-stages of *B. malayi* to identify the tissue target of vaccine action.

Methods Recombinant DIM-1bm (rDIM-1bm) was prepared and antibodies were raised in BALB/c mice. Immunoblots of SDS-PAGE resolved *B. malayi* infective 3rd stage larvae (L_3) and adult worm antigens and rDIM-1bm were prepared and reacted with anti-rDIM-1bm sera. Sections of adult female worms and whole-mount preparations of L_3 and microfilariae (mf) were stained by immunofluorescence using rDIM-1bm antibodies and Alexa Fluor 488 labeled secondary antibodies, and examined under a confocal microscope.

Results Immunofluorescence staining showed that DIM-1bm is localized mainly in the subcuticular muscle layer in the L_3 and the adult worms; no fluorescent signal could be detected in mf.

Conclusion The localization of DIM-1bm in the parasites' muscle layer suggests that the immunoprophylactic efficacy of DIM-1 is evidently due to immobilization of the parasite and its subsequent immune elimination.

Keywords *Brugia malayi* · Disorganized muscle protein-1 · Immunolocalization · Confocal microscopy · Immunoblotting

Lymphatic filariasis (LF), a vector-borne disease caused by the nematode parasites *Wuchereria bancrofti, Brugia malayi* and *B. timori* and transmitted by mosquitoes is one of the world's most debilitating diseases prevalent in tropical and subtropical countries. During a blood meal of mosquito, the infective 3rd stage larvae (L_3) of the parasite carried by mosquito enter the host and develop into adults which produce thousands of microfilariae (mf). The mf circulating in host's blood enter mosquito during another blood meal and develop into L_3. The adult worms have a long life span and produce the clinical and pathological manifestations of the infection. Administration en masse of three antifilarials: diethylcabamazine, ivermectin, and albendazole, to the population in endemic countries is currently the only measure available to contain the transmission of the infection [1, 2], but there is re-emergence of infection in some areas [3] especially in Sri Lanka [4]. There is, therefore, a need for alternative strategies to complement these efforts such as the development of agents that can kill the L_3 and/or the adult worms [5, 6] or a vaccine based on L_3 or adult molecules [7, 8]. In the area of vaccine development, we recently identified a series of products from adult worms of *B. malayi* [9–11] of which 3 proteins/molecules disorganized muscle protein-1 (DIM-1) [12], troponin 1 (Tn1) [13] and Calponin [14] showed remarkable prophylactic potential. DIM-1 is necessary for maintaining body wall muscle integrity in nematodes, including the filarial parasites. DIM-1 of *B. malayi* (DIM-1bm) has almost complete lack of homology with the human counterpart. The importance of the other proteins Tn1 and Calponin is described elsewhere [13, 14]. The present study is focused on studying the localization of

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DIM-1 in the life-stages of *B. malayi* to identify the tissue target of vaccine action.

Laboratory-bred BALB/c mice ($n = 10$; 6–8 weeks old; 20–22 g body weight) were procured from National Laboratory Animal Centre (NLAC), CSIR-Central Drug Research Institute (CSIR-CDRI; kind curtsey of the Head, Parasitology Division, CSIR-CDRI, Lucknow), and used for raising antibodies against recombinant DIM-1 of *B. malayi* (rDIM-1bm).

The different life stages of *B. malayi:* L_3, maintained in *Aedes aegypti* mosquitoes, adult worms and mf maintained in jirds (*Meriones unguiculatus*) [15], were provided by the Head, Parasitology Division, CSIR-CDRI, Lucknow.

Freshly isolated L_3 and adult worms were washed (3 ×) with sterile phosphate-buffered saline (PBS; 0.01 M; pH 7.2) and their soluble somatic extracts were prepared in sterile PBS as described by Chandra et al. [16], Tandon et al. [17] and Dixit et al. (2004) [15] with some modifications. Briefly, 20 adult worms (female: 15; male: 5)/100 µL and 500 L_3/100 µL in sterile PBS, were homogenized in a Porter Elvejhm tissue grinder (A. Thomas Scientific, Philadelphia, PA) for 5 min at 4 °C. The homogenates were subjected to pulsed sonication (Soniprep 150, UK) in cold (4 °C) at 10 kc per second using 9–10 pulses of 20–30 s duration each, followed by centrifugation at 5000 rpm for 5–7 min at 4 °C. The protein content of the preparations was estimated [18], and the preparations were stored in small aliquots at − 80 °C till use.

The gene sequence of DIM-1 of adult *B. malayi* was cloned in TA vector, subcloned in pTriEx-4 expression vector and the rprotein was expressed in BL21-DE3 cells. The affinity purified rDIM-1bm eluted by 300 mM imidazole was resolved as a single band of ~ 40 kDa [12]. The protein was divided into aliquots and stored at − 80 °C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of L_3 and adult worm extracts, and rDIM-1bm protein was carried out using Vertical Dual Mini Gel System Size 2 (8 cm × 7 cm; GeNei Laboratories Private Limited, Bengaluru, India) as described by Laemmli [19] and Dixit et al. [15] using 10% resolving gel. Equal amount of L_3 and adult worm extracts or rDIM-1bm was mixed with an equal volume of sample buffer/protein loading buffer [2 × solution containing Tris buffer (pH 6.8), SDS, β-mercaptoethanol, Glycerol and 0.05% bromophenol blue] separately, followed by heating in boiling water bath for 5 min. Each lane received even loading of 40 µg protein in 20 µL and the proteins were resolved by SDS PAGE. Prestained molecular weight marker (SDS7B; Sigma-Aldrich, St. Louis, USA) was also run simultaneously. Two such sets of gel with resolved proteins were prepared. One set of the gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, USA) in 40% methanol and 10% acetic acid in triple distilled water (de-staining solution) overnight with gentle shaking and then washed with de-staining solution several times till the bands were differentiated from the background.

Parasite extracts and rDIM-1bm protein resolved in the second set of gel were transferred to PVDF membrane (0.22µ, Millipore, India) using a wet Electroblotter (Complete System-Mini Wide; GeNei Laboratories Private Limited, Bengaluru, India) following the method of Towbin et al. [20] and Joseph et al. [21]. The membranes were stored at 4 °C until used.

The method of immunization of animals was as described by Verma et al. [14]. Briefly, groups of BALB/c mice were immunized with three subcutaneous (s.c.) injections of rDIM-1bm in PBS ($n = 5$) or PBS only ($n = 5$; control) mixed with Freund's complete adjuvant/Freund's incomplete adjuvant (FCA/FIA; Sigma-Aldrich, USA). The 1st injection of rDIM-1bm (8 µg/animal) or PBS was given mixed with FCA on day 0, whereas the two booster doses i.e., 2nd and 3rd injections of rDIM-1bm (4 µg/animal) or PBS, were given mixed with FIA on days 14 and 21 post first immunization (p.f.i.). One week after the 3rd injection, blood was collected from the mice and serum was separated and pooled sera were stored in aliquots at − 80 °C till use.

PVDF membrane to which proteins of parasite extracts and rDIM-1bm protein were electrotransferred from gels (see above) were incubated first with 5% skimmed milk (SM; blocking solution) in Tris-buffered saline (TBS; pH 7.4) and then in pooled rDIM-1bm antiserum (1:500 dilution in SM), overnight at 4 °C. The unbound antibodies were removed by washing with TBS-T (containing 0.05% Tween-20). For controls, pooled sera of non-immunized animals were used in place of rDIM-1bm antiserum. The blots were then incubated with HRP-conjugated anti-mouse IgG (1:1000 dilution; Sigma-Aldrich, USA) for 2 h at room temperature followed by TBS-T washes. The blots were developed in chromogenic substrate solution (3,3-Diamino benzidine in TBS + H_2O_2). The molecular weight of immunoreactive molecules in western blots was determined by using Quantity One® (Biorad, USA).

Immunofluorescence staining and confocal microscopy were performed broadly as described by Katta et al. [22]. L_3, mf and adult worms were fixed in cold (4 °C) PBS-buffered 4% paraformaldehyde (PFA; pH 7.4). Mf and L_3 were washed in PBS, transferred to poly-L lysine coated slides and dried at room temperature. PFA fixed adult worms were washed in PBS, dehydrated and embedded in paraffin, sectioned at 5 µm thickness and the sections were mounted on slides and dried. Both deparaffinized sections and the mf/L_3 smears on poly-L lysine coated slides were incubated in blocking solution (BS; 3% BSA in PBS containing 0.02% NaN3, 0.5% glycine; pH 7.2) for 2–3 h at room temperature followed by incubation with rDIM-1bm antiserum (1:20 in BS), overnight at 4 °C. After washes in BS, they were incubated in Alexa Fluor 488 labeled goat anti-mouse IgG,

(1:1000; Thermo Fisher, USA) for 2 h at 4 °C in the dark. The preparations were finally washed in PBS and mounted in 50% glycerine in PBS and stored at 4 °C in the dark till imaging. Sections and smears of the parasites incubated with sera of non-immunized animals in place of rDIM-1bm antiserum served as controls. Immunofluorescence signals in the parasites were imaged using Zeiss LSM 510 META confocal microscope (Zeiss, Jena, Germany) equipped with 20 × /0.75 (and 63 × /1.4) Plan-Apochromat objective and the images were stored to disk for further analysis. Control and test samples were imaged at identical laser and gain settings.

Western blot analysis showed DIM-1 as a single ~ 40 kDa band in the L_3 and adult worm stages of the parasite (Fig. 1).

Confocal microscopic images of adult female worm sections showed DIM-1 signal (green) in the body wall muscle of the parasite and in developing embryos in the female worms (Fig. 2b). The signal was also clearly seen in the body wall muscular layer of L_3 (Fig. 2c). The specificity of the localization was confirmed by the absence of the green signal when rDIM-1bm antiserum was replaced with non-immunized serum (Fig. 2a). In the present study, DIM-1 was found localized mainly in the subcuticular muscle layer of L_3 and adult stage of the female which are the most important life stages of the parasite responsible for infection and pathology. In contrast, we found DIM-1 is absent in mf (data not shown). As in *B. malayi,* DIM-1 was also reported to be localized in the body wall muscles of another nematode *Caenorhabditis elegans*. In this nematode, DIM-1 was

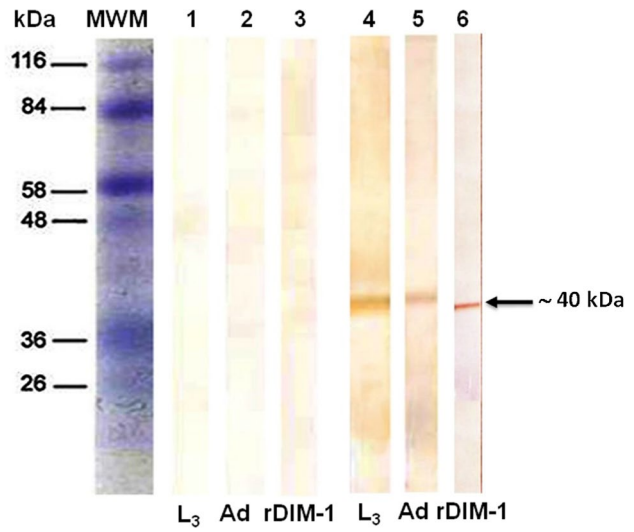

Fig. 1 Immunoblot of SDS-PAGE resolved fractions of L_3 (lanes: 1, 4), adult worm (lanes: 2, 5) antigens and rDIM-1 of *B. malayi* (lanes: 3, 6) reacted with pooled sera of animals. Lanes 1, 2, 3 were reacted with pooled non-immune sera (control) while lanes 4, 5, 6 were reacted with pooled rDIM-1bm immune sera. L_3: Infective 3rd stage larva, Ad: adult worms and rDIM-1

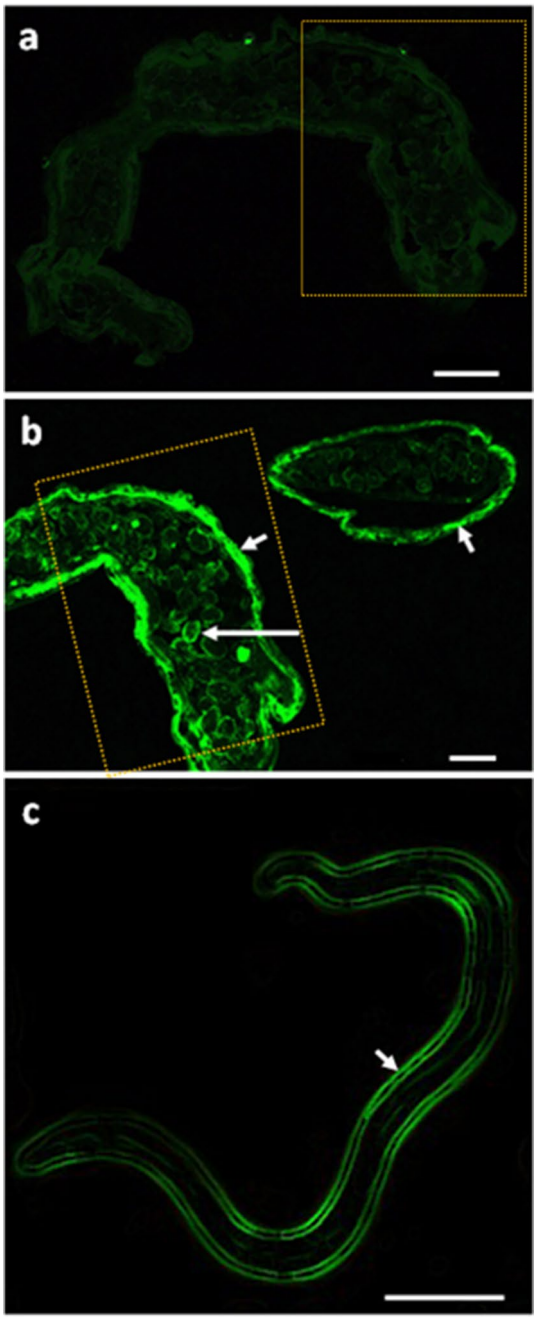

Fig. 2 Confocal microscope images of two serial sections of adult female worm (**a, b**) and unsectioned whole L_3 (**c**) of *Brugia malayi* showing immunofluorescent localization of DIM-1. Fluorescent (Alexa 488) signal of DIM-1 was localized in the subcuticular muscle layers in both L_3 and adult female worms (short arrows) and in the developing embryos in the female worms (long arrow). Specificity of the signal was confirmed by its absence in sections exposed to non-immune pooled sera in place of anti-rDIM-1bm antibody (**a**). The dotted-line rectangles 'a' and 'b'show identical areas in the serial sections. (Scale bar: 50 µm)

localized between and around dense bodies (which are the structures that anchor the actin filaments) of the body wall muscle sarcomeres [23, 24]. The primary role of DIM-1 is to stabilize the thin filament components of the sarcomere and maintaining a strong connection between the myofilament lattice and the basal cell membrane during growth. DIM-1 is thus an important protein necessary for locomotion of the nematode. A homology search of GeneBank showed that *B. malayi* DIM-1 also closely resembles As37 (a 37 kDa protein) localized in the body wall muscles of another nematode parasite *Ascaris suum* [25]; like DIM-1, As37 was also found necessary for motility of the parasite.

The reasons for absence of DIM-1 in the mf stage of *B. malayi* are not clear. It is probably related to immature musculature in mf. Mf are motile vermiform eggs or very young (1st stage) larvae in diapause. The musculature of mf is in the very early stage of formation, with four submedian longitudinal files of muscle cells and one mesenchymal cell, the R1 cell. Formation of definitive musculature in which the R1 cells play an important role, occurs only after mf enter the mosquito vector [26]. It is, therefore, likely that DIM-1 is not expressed in the 'immature' muscle cells of mf but may start expressing during the development of mf into L_3 in the mosquito. Functional in vivo studies using gene silencing by RNAi [27, 28] and gene expression studies may help in testing this hypothesis. However, for the present, the absence of DIM-1 in mf is largely inconsequential, since immunization with rDIM-1bm protected the host against the infection by reducing the worm load [12] and elimination of adult female worms would invariably lead to prevention of microfilaraemia.

In conclusion, DIM-1 was localized in the body wall muscles of L_3 and adult parasites. This suggests that the immunoprophylactic effect of DIM-1 reported earlier by us is evidently via immobilization of the parasite and its subsequent immune elimination.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC), CSIR-CDRI, Lucknow, India, following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environments and Forests, Govt. of India, New Delhi, for use and handling of animals. The animals used in the immunization experiments were housed in climate-controlled pathogen-free animal quarters (Temp.: 23 ± 2 °C; RH: 60%; and photoperiod: 12 h light–dark cycles) at NLAC, CSIR-CDRI, Lucknow, India, and they were fed standard rodent maintenance diet and water ad libitum. The animal study protocol was approved by IAEC (IAEC/2011/145 renewed on 03/07/2014).

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