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Identification of *in vivo* released products of *Onchocerca* with diagnostic potential, and characterization of a dominant member, the OV1CF intermediate filament

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ABSTRACT

A sensitive and specific test for the routine diagnosis of active *Onchocerca* infection is currently lacking. A major drawback in the development of such a test has been the paucity of knowledge of suitable parasite antigens that can serve as targets in antigen-detection assays. In the present investigation, we employed mass spectrometry, bioinformatics and molecular techniques to identify and characterize several potentially diagnostic *Onchocerca* antigens in the *in vivo* nodular fluid, which is being investigated for the first time. The majority of the 27 identified antigens lacked a secretory signal. One of them, also identified and characterized in greater detail with the aid of previously developed monoclonal antibodies (Mabs), was a dominant circulating cytoplasmic intermediate filament protein, previously identified and named, OV1CF. Although OV1CF lacks a secretory signal in its amino acid sequence and is not detected in the pure 42 h *in vitro* released products, it is easily detected in the *in vivo* nodular fluid. We conclude that these *in vivo* released products offer promise as diagnostics markers in onchocerciasis.

1. Introduction

Human onchocerciasis (river blindness), the second leading infectious cause of blindness globally, currently infects an estimated 35 million people in endemic countries of tropical Africa, Latin America and the Arabian Peninsula (TDR, 2005). The skin snip test, which has been considered the gold standard in the diagnosis of the infection (Bradley and Unnasch, 1996), is unfortunately of low sensitivity, especially in areas of low endemicity and in areas receiving mass ivermectin chemotherapy. Numerous antibody-capture tests, many of them based on IgG4 detection, have been developed to replace the skin snip test, but these cannot distinguish clearly between microfilaria positive individuals and uninfected individuals who have been exposed to third-stage larvae (L3). Accurate diagnosis of *Onchocerca volvulus* infection through antigen-capture tests is required for proper patient management and in the evaluation of the successes of control measures. The development of a number of such tests has been attempted (Schlie-Guzman and Rivas Alcala, 1989; Mbacham et al., 1992; Cho-Ngwa et al., 2005; Wembé et al., 2005), but none of these has yielded the required sensitivity and specificity. Wembé et al. (2005) developed an antigen-detection test that was highly sensitive, but the evaluation of the specificity of their polyclonal antibody-based test using body fluid from patients with related nematode infections is yet to be done. The lack of a definitive solution to this problem has in part been due to the lack of knowledge of defined parasite antigens that are abundantly released into host's circulation, and that can thus serve as reliable markers of active infection. If such an antigen is fairly stable and polymeric or contains repeating structural units, it could allow the configuration of a highly sensitive and specific sandwich immunoassay using a single monoclonal antibody. The presence of circulating *O. volvulus* antigens in patients' body fluid was first demonstrated in the early 1980s by a number of workers (Paganelli et al., 1980; Ouaissi et al., 1981).

Intermediate filaments (IFs) are cytoskeletal protein superstructures formed by members of a family of related proteins.

Abbreviations: OOTE, *O. volvulus* total extract; ESP, excretory/secretory products; Mab, monoclonal antibody; IF, intermediate filament; IFs, intermediate filaments; NRP, nodular released products; mf, microfilaria; PBS, phosphate buffered saline; FCS, fetal calf serum; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; ACN, acetonitrile; 1D, one dimensional; ppm, parts per million.

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They have a diameter between that of microfilaments and microtubules, and form long polymeric structures. Most types of IFs are located in the cytosol. The intermediate filament (IF) proteins of *O. volvulus* have been cloned from cDNA libraries and characterized serologically in a number of studies (Seeber et al., 1994; Zhang and Miller, 1994, 1995; Chandrashekar et al., 1995). Chandrashekar et al. (1995) characterized the circulating IF protein, OV1CF for its role in the mediation of autoimmune keratitis (onchokeratitis), and also reported that it is an excretory/secretory product since it was detected among the parasite excretory/secretory products. In our laboratory, three monoclonal antibodies (Mabs), termed UB1, UB6 and UB7 were developed and shown by Western blotting to react strongly with a set of the same 15–22 polypeptides in the crude extracts of *Onchocerca ochengi* (Cho-Ngwa et al., 2005). These polypeptides were shown earlier in this study to be fragments or polymers of OV1CF, demonstrated to be a prominent *in vivo* released product of *Onchocerca*. In the present study, we used mass spectrometry to identify several *in vivo* released products of *Onchocerca*, one of them being OV1CF. We additionally characterized OV1CF further to demonstrate the usefulness of these parasite released antigens as targets in antigen-capture diagnosis of active *O. volvulus* infection.

2. Materials and methods

2.1. Parasites and nodular released products (NRP)

Intact *O. ochengi* adult female and male worms were obtained as nodular masses by careful dissection of nodules still attached to the bovine skin using a sterilized razor blade (Cho-Ngwa et al., 2007). During the dissection, a small incision was first made into the nodule such that the blade did not cut the worms inside. The nodular mass was then carefully squeezed and scraped out of the surrounding connective tissue, such that the sharp edge of the blade was kept away from the worms as much as possible. Thus, it was usual for some host connective tissue to be recovered with the worms (as nodular masses). The integrity of the adult worms was checked by observing them with a dissecting microscope. The worms were recovered from the nodule within 60 min of slaughtering of the infected cows. Working at 4 °C, the entire nodular masses of 52 (total of 1.3 g wet weight) fresh *O. ochengi* nodules were submerged in serum-free RPMI-1640 culture medium, then agitated gently (35 cycles/min) for 30 min and finally centrifuged at $5000 \times g$ for 15 min. The supernatant obtained was labeled nodular released products (NRP) and saved at −70 °C until used. For the preparation of clean *O. ochengi* worms, the extracted nodular contents were further digested with collagenase B as previously described (Cho-Ngwa et al., 2007). Microfilariae (mfs) were prepared essentially as described by Bianco et al. (1980). Briefly, a piece of infected umbilical bovine skin was washed, sterilized with 70% ethanol and then skin slivers were obtained and placed in culture medium for viable mfs to emerge. The latter were washed twice and concentrated by centrifugation.

2.2. O. ochengi in vitro excretory/secretory products (ESP)

The excretory/secretory products of *O. ochengi* adult worms were obtained as previously described (Cho-Ngwa et al., 2007). Briefly, intact adult worms (males and females, with some of the females harboring mfs) obtained after the collagenase B digestion step were washed 5 times with copious amounts of fresh culture medium. The adult worms were cultured for 42 h at 37 °C and 5% CO_2, and at 85 mg wet weight per ml of serum-free RPMI-1640 culture medium to obtain total *O. ochengi* ESP (ESPt). The mfs were cultured similarly at 2×10^5 mfs per ml of culture medium to obtain the mfs ESP (mfESP). All the ESPs were cleared by centrifugation at $4000 \times g$ for 15 min and the supernatants were saved at −70 °C until used.

2.3. Parasite crude extracts

O. volvulus worms and the total extract (OVTE) were prepared essentially as previously described (Titanji et al., 1985). Briefly, nodules extirpated from patients were digested with collagenase and the recovered adult male and female worms were homogenized in buffers of different ionic strength and pH. The supernatants obtained after centrifugation were pooled to obtain the total extract. Part of the extracted adult worms were also placed in culture medium and the males, identified by their much smaller sizes, were separated from the females by picking. Sixty (60) male worms were thoroughly sonicated in 500 μl of phosphate buffered saline (PBS, pH 7.4) on ice blocks using a Vibrasell VC50 sonicator (Sonic and Materials, USA) following the manufacturer's instructions. The homogenate was centrifuged at $4000 \times g$ to obtain the supernatant which was labeled as male worm sonicate (mson). The mfs sonicate was prepared similarly by sonicating about 5×10^5 mfs in 500 μl of PBS (pH 7.4). Microscopic examinations were done to ensure that the sonications were effective. Total protein concentrations were estimated by the Bradford method (Ausubel et al., 1988).

2.4. 1D gel electrophoresis and in-gel digestion of proteins

Proteins were separated on pre-made 4–12% gradient NuPAGE® bis–tris gels (Invitrogen, CA, USA) using MOPS™ running buffer according to the manufacturer's recommendations. Standard protein size markers (Invitrogen, CA, USA) were run in parallel. Gel lanes were washed twice in pure water for 15 min and then cut into small slices at the positions 30K, 60K, 90K and 180K. Proteins were reduced by incubating the gel bands in 10 mM DTT/100 mM Ambic (ammonium bicarbonate) solution at 56 °C for 1 h. Then the proteins were carboxyamidomethylated with 55 mM iodoacetamide/100 mM Ambic for 1 h at room temperature in the dark. Enzymatic digestions were performed by adding sequencing grade porcine trypsin (1:50, Promega, Madison, WI) and incubated at 37 °C overnight. The tryptic peptides were extracted three times with 200 μl of ACN/water (1:1) solution. Combined extracts were completely dried in speed vacuum, resuspended in 50 μl of 0.1% formic acid and then stored at −20 °C until used for analysis by LC–MS/MS.

2.5. LC–MS/MS analysis

The peptide samples obtained from proteolytic digestion were analyzed on an Agilent 1100 capillary LC (Palo Alto, CA) interfaced directly to a LTQ linear ion trap mass spectrometer (Thermo Electron, San Jose, CA). Mobile phases A and B were H_2O/0.1% formic acid and ACN/0.1% formic acid, respectively. The peptide samples were loaded for 50 min using positive N_2 pressure on a PicoFrit 8-cm by 50-μm column (New Objective, Woburn, MA) packed with 5-μm-diameter C_{18} beads. Peptides were eluted from the column into the mass spectrometer during a 60 min linear gradient from 5% to 60% of total solution composed of mobile phase B at a flow rate of 200 μl min^{-1}. The instrument was set to acquire MS/MS spectra on the nine most abundant precursor ions from each MS scan with a repeat count of 1 and repeat duration of 5 s. Dynamic exclusion was enabled for 200 s. Raw tandem mass spectra were converted into the mzXML format and then into peak lists using ReAdW software followed by mzMXL2Other software (Pedrioli et al., 2004). The peak lists were then searched using Mascot 2.2 (Matrix Science, Boston, MA). The sampling in the mass