Ivermectin disrupts the function of the excretory-secretory apparatus in microfilariae of *Brugia malayi*

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Ivermectin (IVM) is a broad-spectrum anthelmintic used in filariasis control programs. By binding to nematode glutamate-gated chloride channels (GluCls), IVM disrupts neurotransmission processes regulated by GluCl activity. IVM treatment of filarial infections is characterized by an initial dramatic drop in the levels of circulating microfilariae, followed by long-term suppression of their production, but the drug has little direct effect on microfilariae in culture at pharmacologically relevant concentrations. We localized *Brugia malayi* GluCl expression solely in a muscle structure that surrounds the microfilarial excretory-secretory (ES) vesicle, which suggests that protein release from the ES vesicle is regulated by GluCl activity. Consistent with this hypothesis, exposure to IVM in vitro decreased the amount of protein released from microfilariae. To better understand the scope of IVM effects on protein release by the parasite, three different expression patterns were identified from immunolocalization assays on a representative group of five microfilarial ES products. Patterns of expression suggest that the ES apparatus is the main source of regulated ES product release from microfilariae, as it is the only compartment that appears to be under neuromuscular control. Our results show that IVM treatment of microfilariae results in a marked reduction of protein release from the ES apparatus. Under in vivo conditions, the rapid microfilarial clearance induced by IVM treatment is proposed to result from suppression of the ability of the parasite to secrete proteins that enable evasion of the host immune system.

filarial nematode | macrocyclic lactone | glutamate-gated chloride channels

Infections with filarial nematodes are not only major causes of long-term disability, but also create economic hardship and social stigma in endemic areas. In recent years, very significant efforts toward elimination are in place for two of these diseases: lymphatic filariasis (LF), caused by infection with *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori*, and *Brugia pahangi*, present in 83 countries with ∼120 million people infected (1); and onchocerciasis (river blindness) caused by *Onchocerca volvulus,* predominant in 22 countries in sub-Saharan Africa, with ∼18 million currently infected (2).

Filarial infections are chronic; adults and microfilariae (mf) can survive from years to decades in the mammalian host. Human infection starts with the release of infective larvae (L3) during feeding of the arthropod vector. L3 enter the host at the puncture site and migrate for maturation to lymphatic vessels (*W. bancrofti* and *Brugia* spp.), or to the subcutaneous and connective tissues (*O. volvulus*). After mating, female worms can release hundreds to thousands of mf per day. The mf from lymphatic filariae transit from lymph to the peripheral circulation; *O. volvulus* mf usually remain in the skin and lymphatics of connective tissue, where mf become available for ingestion by the vectors.

No vaccine is available; filariases are controlled through chemotherapy, although vector control and bed-nets are important adjuncts. Current programs for eradication of these diseases include mass drug administration (MDA) of a microfilaricide to interrupt transmission and diminish morbidity. Control of onchocerciasis since the late 1980s has primarily relied on MDA programs with ivermectin (IVM). By 2005, more than 407 million treatments had been administered for this disease (3) (> 800 million by 2009). LF treatment includes annual, single-dose combinations of either IVM or diethylcarbamazine with albendazole (ABZ). More than 1.9 billion treatments have been provided since 2000 through the Global Program for Elimination of LF (1, 4).

IVM, a macrocyclic lactone endectocide, is considered a microfilaricide because of the fast mf clearance observed and the extremely prolonged suppression of their reappearance after dosing (5). Although kinetics of mf clearance vary depending on the species, most of the dramatic changes in the mf load take place during the first week of treatment. In LF, 100 to 200 μg/kg IVM reduces blood mf density to <5% and to 32–39% of the initial mf load in Bancroftian and Brugian filariasis, respectively, 1 d after dosing (6, 7). In onchocerciasis, reduction in mf numbers 1 d after a dose of 150 μg/kg is ∼60% of pretreatment level (8). One week after treatment, microfilaremia is reduced to ∼0% in Bancroftian filariasis and onchocerciasis and ∼10% in Brugian filariasis; these reductions are sustained for at least 6 mo.

In contrast to the unquestionable efficacy of in vivo IVM treatment and despite its extensive use, the pharmacological mechanism underlying rapid mf clearance remains elusive. In vitro, IVM effects on mf appear only at concentrations far above levels achieved during therapy for filariases (9). In the model nematode *Caenorhabditis elegans* and intestinal parasitic nematodes, IVM affects pharyngeal pumping and motor activity at concentrations 0.1 to 10 nM (10–12). Consistent with these effects, glutamate-gated chloride channels (GluCls), the primary nematocidal targets for IVM, are localized in the motor nervous system of these organisms, particularly in motor neuron commissures, lateral and sublateral nerve cords, amphids, and pharyngeal neurons (13).

GluCls are members of the "Cys-loop" family of ligand-gated ion channels, which form pentameric structures composed of one or more subunit types (14). GluCl gene diversity across the phylum Nematoda is significant, but the biological processes regulated by the activity of these channels have been investigated in few species. Neither their localization nor physiological roles can be simply extrapolated between phylogenetically distant nematode species. In *C. elegans*, for example, six GluCl genes have been identified as encoding eight possible protein subunits formed by alternative splicing (14). In contrast, homology searching for gene models that represent probable GluCl subunits in the genome of *B. malayi*

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reveals only four subunits, two of them (AVR-14A and AVR-14B) related to IVM-sensitive channels (15, 16).

Although direct evidence is lacking, a synergistic effect between IVM and the host immune system has been proposed to explain the clearance of mf (9). One possibility is that IVM disrupts filarial processes that result in the modulation of the host immune system. The immunomodulatory ability of filarial nematodes generates a host phenotype characterized by suppression of both Th1 and Th2 responses, impaired proliferation of T cells, increased production of the regulatory cytokine IL-10, and higher levels of IgG4 (17). However, the molecular mechanisms that enable parasite evasion of host immune responses are incompletely understood. It appears that most immunomodulatory strategies used by these parasites are based on soluble mediators, especially proteins that modify or degrade cellular or molecular components of the host immune system (18).

Our goals in these experiments were to obtain a better understanding of the mechanism of action of IVM in mf, focusing on inhibition of the secretion by the parasite of immunomodulatory proteins. We conducted experiments to identify the anatomical location of GluCl expression in *B. malayi* mf. Localization was compared with the anatomy of the parasite excretory-secretory (ES) system. Finally, we directly measured the effects of IVM on protein secretion by mf.

Results

Identification of the Putative IVM Receptors in *B. malayi*: Cloning of AVR-14 Subunits. A homology search revealed four possible GluCl subunits in *B. malayi* (16). Phylogenetic analysis and naming according to Beech et al. (19) allowed us to infer that these subunits are orthologs of the GLC-2, GLC-4, AVR-14A, and AVR-14B subunits in *C. elegans* and other nematodes. Among them, only AVR-14A and AVR-14B are reported to form IVM-sensitive channels (14). Because assembly and annotation of these subunits in the *B. malayi* genome database was ambiguous and incomplete, the full ORFs for these subunits were assessed (*SI Materials and Methods*, Fig. S1, Table S1).

A combination of PCR and 5′ RACE was used to clone full-length sequences for two splice variants of the *Bma-avr-14* gene. These sequences encode 419 and 427 amino acids of the Bma-AVR-14A and Bma-AVR-14B subunits, respectively. Bioinformatic analysis predicts the characteristic features of the cys-loop ligand-gated ion channels gene family (Fig. S2), which include a long N-terminal extracellular domain containing a disulphide-bond formed by two conserved cysteine residues separated by 13 amino acids, and four transmembrane regions (TM1–TM4) with a large cytoplasmic loop between TM3 and TM4 (20). Phylogenetic analysis using neighbor joining trees retained clustering of both Bma-AVR-14 subunits with their respective orthologs in nematodes (Fig. 1).

Bma-AVR-14 subunits were more closely related to AVR-14 subunits from the filarial nematodes *Dirofilaria immitis* and *O. volvulus* than to those of other nematodes (Fig. 1), with amino acid sequences 94 and 95% identical to their respective homologs in *D. immitis*. Our results agree with previous findings that the alternative splice patterns of filarial species differ from those of *C. elegans* and *Haemonchus contortus* (21). Filarial *avr-14* transcripts were characterized by the presence of the SL1 sequence at the 5′ end followed by a shared sequence containing the coding sequence for the N-terminal region of the two subunits and different C-terminal coding sequences. Filarial *avr-14A* transcripts lack the long UTR upstream of the poly-A tail characteristic of the *avr-14A* transcripts from *C. elegans* and *H. contortus* (Fig. S1). These differences reflect the considerable phylogenetic distance between filarial nematodes and the other two species (21).

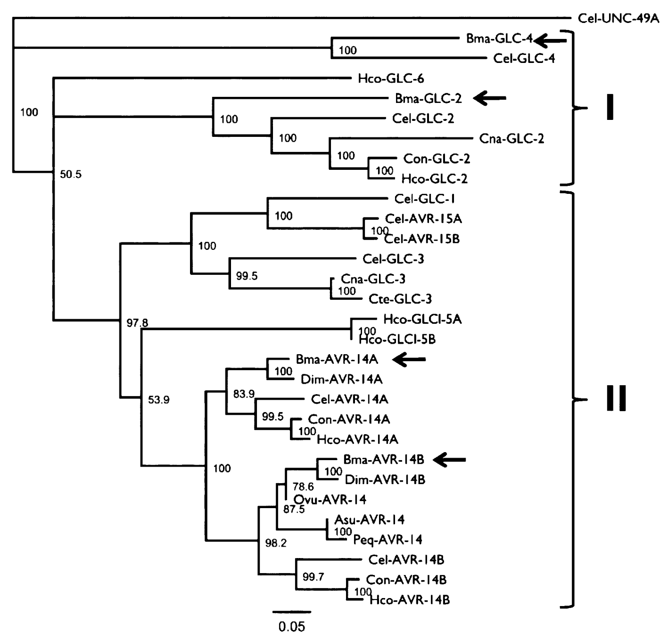

Fig. 1. Boot-strapped (1,000) neighbor-joining tree generated from a ClustalX alignment of the Bma-AVR-14A and -B sequences and sequences of GluCls from other parasitic nematodes and *C. elegans*. Outgroup sequence for this tree was set with a γ-aminobutyric acid receptor subunit from *C. elegans* (Cel-UNC-49A). I, nonsusceptible or unknown susceptibility to IVM; II, susceptible to IVM.

Localization of GluCl Subunits in *B. malayi* Reveals Association of Muscle with the mf ES Vesicle. To localize GluCl expression in mf, an antibody was raised against the peptide LRTKMILR-REFS-cysteine, mapping to aa 239–250 of Bma-AVR-14A. Cross-reactivity of the affinity-purified antibody against the peptide ARVMLLLRREYS-cysteine, corresponding to the same region in Bma-AVR-14B, was determined by ELISA. The antibody has approximately fivefold higher affinity for the Bm-AVR-14A subunit compared with the Bma-AVR-14B subunit. Comparison with the same region of the other two *B. malayi* GluCl subunits showed a comparable level of similarity to that between Bma-AVR-14A and -B. We assume that the immunolocalization protocol employed here does not necessarily discriminate between the different GluCl subunits.

B. malayi GluCl subunits were localized by confocal laser-scanning microscopy, with counterstaining against nuclei and actin (Fig. 2). Counterstaining distinguished the major anatomical features of *B. malayi* mf, including the oral opening, the nerve ring, the ES apparatus, the inner body, and anal pore. GluCl subunits were specifically and selectively localized to tissue surrounding the Mf ES vesicle. Ultrastructural observations have described this apparatus in *B. malayi* mf as consisting of a large vesicle that opens to the cuticle and a large excretory cell connecting to the vesicle by a cytoplasmic bridge (22). Actin staining revealed that muscle tissue surrounds the ES vesicle and colocalizes with the GluCl signal (Fig. 2*B*).

The presence of a muscle layer encompassing the ES vesicle suggests the possibility of neuromuscular regulation of the release of ES products. This finding implies that protein transit from the vesicle to the exterior pore of the ES apparatus is an active process. Under this assumption, we hypothesize that GluCls regulate the activity of this muscle and that IVM treatment of mf would lead to a decrease in protein release.

Microfilariae-ES Apparatus Is the Main Source for Protein Secretion. To clarify the contribution of the ES apparatus to protein release from mf, and consequently the role of GluCl subunits in the

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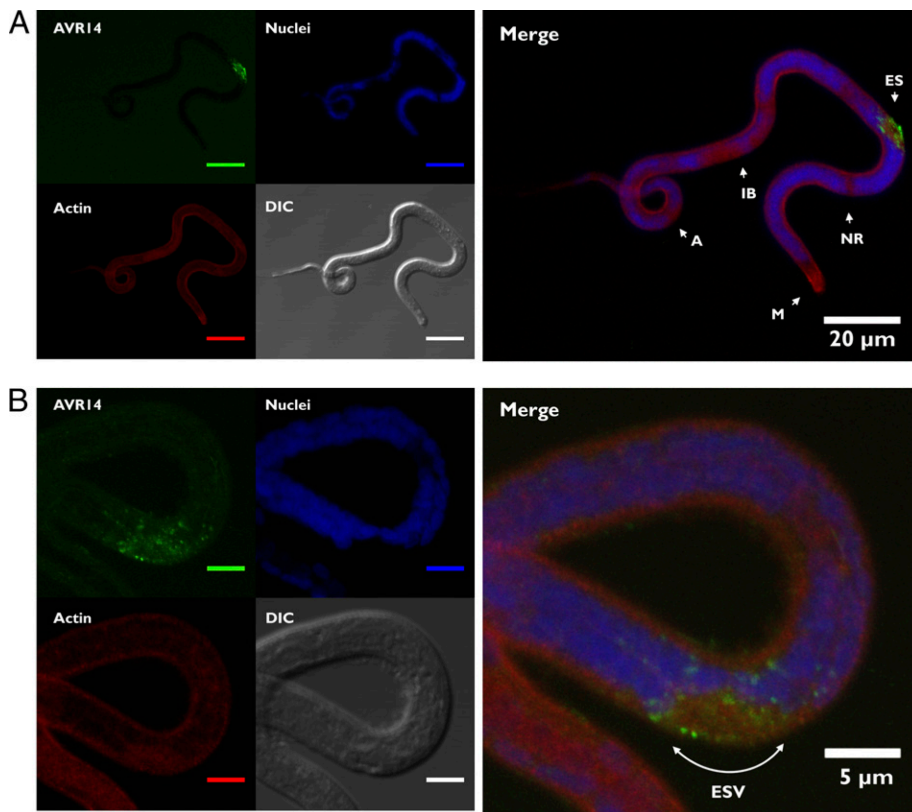

Fig. 2. Immunolocalization of AVR-14 subunits in *B. malayi* mf by confocal laser scanning microscopy. (*A*) Bma-AVR-14 specific signal was detected in proximity to the ES apparatus. Counter staining with DAPI and Phalloidin-Rhodamine distinguishes major anatomical features (A, anus; ES, excretory-secretory apparatus; IB, inner body; M, mouth; NR, nerve ring). (*B*) A closer examination of the signal reveals colocalization of Bma-AVR-14 subunits with a muscle structure surrounding the ES vesicle (ESV).

processes that regulate it, a comparative analysis of the anatomical pathways involved in protein secretion was undertaken. Immunolocalization studies by confocal laser scanning microscopy of proteins known to be released from *B. malayi* mf, termed ES proteins, including translationally controlled tumor protein-like protein (Bma-TCTP), macrophage migration inhibitory factor-1 (Bma-MIF-1), cysteine protease inhibitor-2 (Bma-CPI-2), venom allergen-like protein-1 (Bma-VAL-1), and triose phosphate isomerase (Bma-TPI), established three different localization patterns for ES proteins.

Bma-CPI-2, Bma-VAL-1, and Bma-TPI were exclusively found in the ES apparatus (Fig. 3*A*). Localization was typically characterized by a strong signal in the ES vesicle and pore, suggesting that a mechanical force is involved in retaining proteins in this vesicle (Fig. S3). In some specimens, the entire ES apparatus was labeled, showing the complete secretion path: ES cell–cytoplasmic bridge–vesicle–pore.

A different pattern was identified for Bma-TCTP (Fig. 3*B*). In ~50% of the specimens stained with anti-Bma-TCTP, signal was found in both the ES apparatus and at the inner mf sheath surface. Localization in the sheath was characterized by punctate staining surrounding the whole body. Even for this protein, other mf were stained exclusively in the ES apparatus, similar to the first pattern. No staining in non-ES cells was observed, suggesting that this cell is the unique source of TCTP in the inner sheath and its accumulation on the parasite surface may occur after release from the uterus.

A third staining pattern was observed with Bma-MIF-1 (Fig. 3*C*). This protein was found throughout the midbody from the nerve ring to the inner body, including the ES cell, as well as in the ES vesicle, where a strong signal was observed. Compared with the TCTP staining pattern at the mf inner sheath, the MIF-1 signal was also high toward the lateral axis, but in a different layer, which likely comprises the hypodermis and cuticle and, to a lesser extent, in the inner sheath (Fig. 3 *B* and *C*, *Right*). This distribution suggests that the hypodermal cells in this area may be the main MIF-1 source for protein accumulation in the cuticle and sheath, but that secretion from the ES vesicle is a prominent source of the protein that accumulates in culture media containing mf.

It must be noted that the mf sheath has been shown to be impermeable to antibody penetration during immunostaining, requiring intensive permeabilization to allow antibodies to reach internal antigens. Thus, the mf sheath imposes an additional barrier to lateral protein release. These results suggest that the ES apparatus constitutes the major anatomical pathway for the release of ES products into the external environment.

IVM Treatment of *B. malayi* mf Reduces Protein Release. To probe the relationship between GluCl-regulated muscle activity and protein release from the ES apparatus, the amount of protein released in vitro by mf was quantified in media containing IVM over 72 h in culture (Fig. 4*A*). ABZ was included as a positive control, as it has been shown to reduce protein secretion by the hookworm *Ancylostoma ceylonicum* and other nematodes through inhibition of microtubule polymerization (23) (Fig. 4*B*).

A decrease in protein output was observed as soon as 24 h after IVM exposure. As there were no significant differences among treatments in total protein in mf-somatic extracts at any time-point; it can be inferred that the drop in protein secretion is not related to a simple decrease in mf viability, but instead is consistent with paralysis of the muscle associated with the ES vesicle (Fig. 4*A*). Concentration-dependence of the IVM effect was only observed at the 48- to 72-h time-point after exposure, suggesting that in addition to the rapid and consistent effect of IVM on mf protein secretion, there may be a secondary effect at higher IVM concentrations. Exposure to 0.1 μM IVM decreased protein secretion by 58% ($P < 0.01$), 68% ($P < 0.05$), and 42% ($P < 0.05$) compared with nontreated controls at 24, 48, and 72 h, respectively.

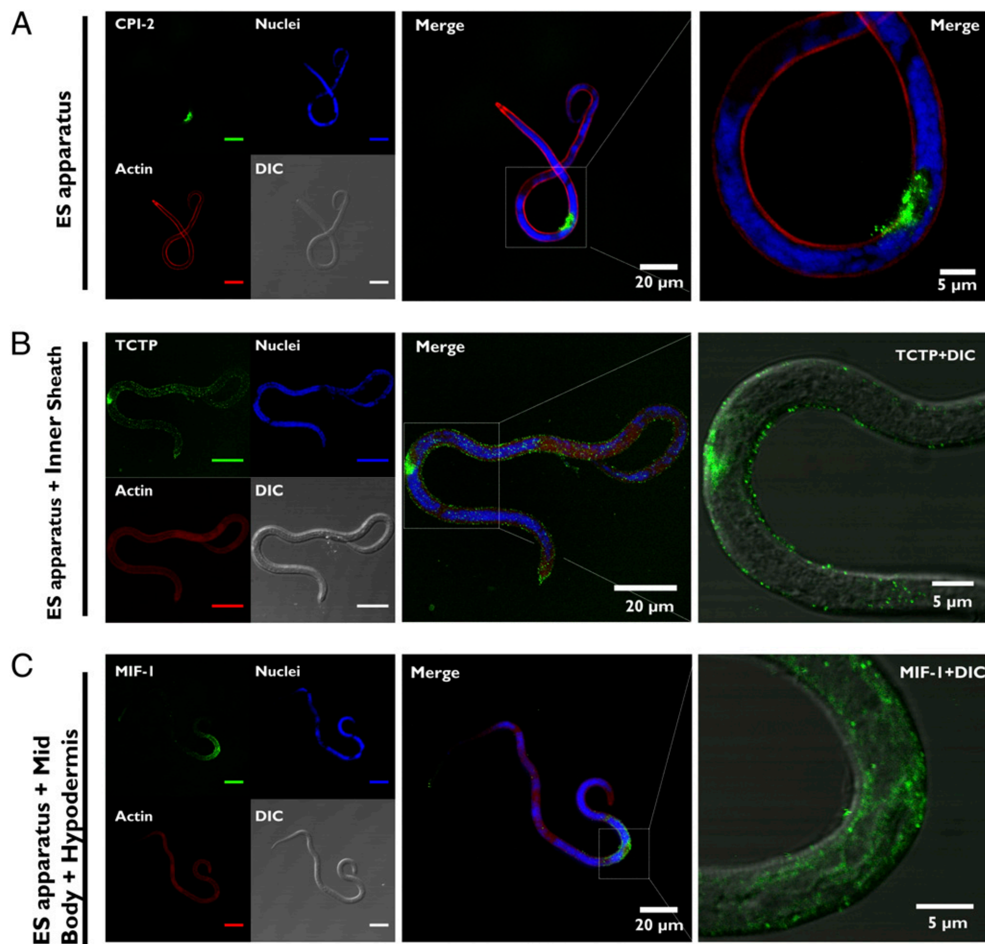

Fig. 3. Immunolocalization studies of ES proteins in *B.malayi* mf reveal three different expression patterns. (*A*) Protein localization exclusively in the ES apparatus. Representative staining is shown for CPI-2. Also exhibiting the same pattern, VAL-1, TPI, and a fraction of mf stained with anti-TCTP (Fig. S3). (*B*) Protein localization in either the ES apparatus or the mf inner sheath. This pattern was exhibited in another fraction of specimens stained for TCTP. (*C*) The third pattern, observed for MIF-1, consisted of prominent expression through the midbody. Images on the *Right* of *B* and *C* are presented as merged images of single planes showing signal presence in the ES-vesicle and differences in compartmentalization of TCTP and MIF-1 toward the parasite surface.

Although limited by protein availability, preliminary analyses using 2D difference gel electrophoresis (2D-DiGE) were done to detect changes in relative protein abundance in mf-ES products upon treatment with 0.1 μM IVM (additional information in *SI Text*, Fig. S4, and Table S2). Protein profiles and relative protein abundance were essentially unchanged, providing additional evidence that the ES apparatus is the main source for protein export.

Discussion

Despite the remarkable reduction in morbidity and profound interruption of disease transmission achieved by the MDA programs for the control of onchocerciasis and LF (1), there are concerns about the feasibility of eliminating filarial infections without the availability of a macrofilaricide in the short term; additional concerns about the possible emergence of resistance to IVM, which forms a mainstay of both programs (23, 24), ex-

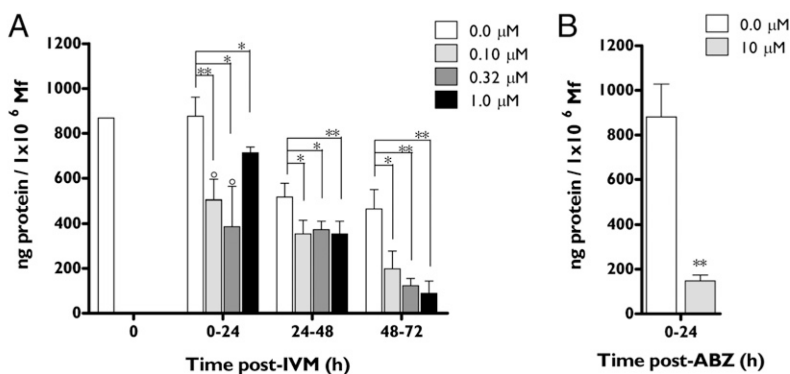

Fig. 4. Effects of two anthelmintic drugs on protein release from *B.malayi* mf. (*A*) IVM reduces protein release in vitro. Mf (2.5×10^5 mf/mL) were incubated from 0 to 72 h in RPMI 1640 with or without IVM. Media exchange was performed each 24 h ($n = 3$) at 24, 48, and 72 h. (*B*) Protein release in vitro at 24 h from mf incubated with or without ABZ (10 μM). Mean ± SD; $*P < 0.05$; $**P < 0.01$, control vs. treatment; $°P < 0.05$, 1.0 μM IVM vs. treatment.

PHARMACOLOGY

acerbate the need to attain a much better understanding of the pharmacology of existing antifilarial drugs, which remains poor (5). Inadequate understanding of the basis for the action of IVM on these parasites impedes our ability to optimize its clinical use and to sustain the efficacy of the drug in the mid- to long-term. Generating new tools for detecting and monitoring filarial infections, including possible IVM-resistant parasite populations, and for discovering and developing new anthelmintics represent critical challenges, as current knowledge on the mechanism of action of IVM does not explain how it alters filarial viability and leads to mf clearance from the host (5).

IVM is a potent and generally very safe drug for the treatment of nematodes, and also infestations of ectoparasites of human and veterinary relevance (25). This remarkably broad spectrum of action and record of mammalian safety is partly a result of the absence of GluCls from vertebrates and the essential role of these channels in neurotransmission in some types of invertebrates (14). Beyond this, the cascade of events following GluCl opening, which leads to the observed effects of IVM treatment in filariases, is still unclear.

In the present work, we assembled the full ORF sequences of two subunits of the *avr-14* GluCl of *B. malayi*. As no additional orthologs of IVM-sensitive channels were found in the *B. malayi* genome database, these proteins must represent the main putative target for IVM in this parasite based on data obtained in other species of nematodes (26–28). Identifying their anatomical localization in mf provides new insights to explain the pharmacological consequences of treatment of filariases with IVM.

The localization of GluCl subunits observed in *B. malayi* has not been reported in other nematodes. The unique presence of GluCl immunoreactivity colocalizing with a muscle structure surrounding the ES vesicle allowed us to build a series of hypotheses about the role of these channels and the effect of IVM in mf. First, it is expected that glutamatergic synapses occur in this area and regulate the neuromuscular physiology that controls relaxation and contraction of this muscle and, so, protein secretion. Second, as no other sites of expression of GluCl subunits were observed, muscle activity in this area of mf must account for the mechanism of action of this drug against this stage. It has been commonly observed that IVM does not affect mf motility or viability at pharmacologically relevant concentrations in culture (5, 9), which is consistent with the apparent absence of GluCl expression in regions associated with somatic muscle function. Recently, it has been reported that IVM concentrations >1 μM affect mf motility (29); this may be associated with drug effects on GABA-gated chloride channels, which are secondary targets for the ML class (30). These effects may partially account for the concentration-dependence of the drug effect on secretion seen at the later time-point. Third, the work done by the ES-associated muscle layer must be exerted on the ES vesicle, allowing secreted proteins to be pumped out through the ES pore. Like the pharynx in adult nematodes, the high internal turgor pressure in nematodes would tend to keep the ES pore closed, so that active contraction against this force would be needed to permit protein expulsion in a regulated fashion. Exposure of mf to IVM hyperpolarizes the muscle, resulting in its paralysis, thus preventing opening of the ES pore and inhibiting protein secretion.

Proteomic analysis of ES products has been limited by the very low amount of protein recovered from in vitro incubations. Overcoming this limitation using highly sensitive approaches has lead to the identification of most of these proteins in *B. malayi* (31–33). However, information about the anatomy and physiology of the protein secretory pathways in filarial nematodes has not been intensively investigated. The comparative analysis of the localization of ES proteins presented here aimed to dissect the sources and routes involved in the generation of these proteins. For this analysis, we selected proteins from the secretome with and without predicted N-terminal secretion signal peptide sequences. This process allows a better understanding of the scope of the effect of IVM on overall protein release. Our results confirmed that, independent of the cellular secretory pathway involved (classic vs. nonclassic), the role of the ES apparatus is central for the delivery of proteins from mf to the host environment.

This conclusion is supported by the fact that all of the proteins accumulated in the ES vesicle. Although it could be argued that lateral secretion across the mf surface can also be a component for protein release, there seems to be no role for a mechanical force helping to deliver protein to the exterior, as in the ES vesicle; the consistent inhibition of secretion of all ES proteins by IVM argues that a single, regulated pathway underlies at least the majority of protein secretion in mf. Coupled with the general impermeability to macromolecules exhibited by the mf sheath, this consideration allows us to infer that the ES vesicle pathway is the predominant route of protein secretion from mf.

Evidence for rapid changes in the host after IVM treatment that may be deleterious to mf has been presented elsewhere. This evidence includes the induction of nitric oxide derivatives in patients infected with *Loa loa* or *O. volvulus* and changes in the circulating levels of chemokines, such as RANTES, IL-8, and IFN-γ, which may affect recruitment of eosinophils (34). The requirement of serum factors to promote in vitro cell-mediated cytotoxicity in combination with IVM has been shown in the *Dipetalonema (Acanthocheilonema) viteae* and *Litomosoides carinii–Mastomys natalensis* systems (35, 36), supporting the argument that IVM treatment in human filariases leads to rapid disruption of the host-pathogen molecular negotiation via suppression of parasite protein secretion.

In support of these hypotheses, mf treatment with IVM in vitro resulted in a marked reduction in protein release. These observations were made in a time-frame that paralleled the reduction in circulating mf induced by IVM in patients with Brugian filariasis and at concentrations that are close to peak therapeutic levels (37). It is therefore reasonable to infer that IVM treatment reduces protein release from mf in vivo. This hypothesis provides insights to explain how mf are rapidly cleared by IVM treatment.

In this context, our results support the proposal that the rapid clearance of mf following treatment with IVM is effectively the result of a direct or indirect interaction with the host immune system (9, 38). The proteins released in vitro by Bma-mf differ from those released by adults and are associated with modulation and evasion of the host immune system, among other potential functions (32, 33). As it is accepted that parasite protein secretion plays a fundamental role in host immunomodulation (18), it is reasonable to infer that inhibition of protein secretion would result in the inability of the parasite to neutralize preexisting and induced host effectors at the local and systemic levels.

Our data do not address the lack of macrofilaricidal activity of IVM observed in vivo. The large size of the adult worms may render them relatively insusceptible to the actions of host immune effector cells and molecules, making the antisecretory effects of the drug irrelevant for this stage. Infectious (L3) larvae and developing (L4) juvenile stages are targeted by IVM, at least in some filariases, and investigations on whether the observations made here on mf can be extended to L3 stages are underway.

Taken together, our results show that IVM targeting of the AVR-14 subunits from *B. malayi* mf in vitro leads to the disruption of protein release from the ES apparatus, the main pathway for protein delivery to the mammalian host. This finding provides additional insights on the mechanism of antifilarial action of IVM in vivo that can be valuable for drug and vaccine development. Finally, our data support a role of ES proteins in modulation and evasion of the host immune response by filarial nematodes.

Materials and Methods

More detailed information on the materials and procedures is found in *SI Materials and Methods*. All animal procedures were approved by the Animal Care Committee of McGill University and were in accordance with the guidelines of the Canadian Council on Animal Care. Infected Mongolian jirds (*Meriones unguiculatus*) were obtained from the Filariasis Research Reagent Repository Center (Athens, GA). Mf and adult *B. malayi* were recovered >120 d postinfection from the peritoneal cavity, as described in ref. 32.

Cloning and Sequencing of Bma-AVR-14 Subunits. Partial sequences for two GluCls (AVR-14) were obtained from the *B. malayi* genome project (Bm1_00335 and Bm1_15450). Completion of the full coding sequences for these genes was achieved by following a strategy like that used for *avr-14* from *D. immitis* (21).

Immunohistochemical Analysis. For immunohistochemical analysis, antibodies were obtained from external sources or raised against on-site produced His-tagged proteins. The mf were fixed, permeabilized, and treated by an adaptation of the tube protocol for *C. elegans* (39). Controls include observation of mf with omission of primary antibody and, for on-site produced proteins, with peptide- or protein-adsorbed primary antibodies.

In Vitro Treatment of *B. malayi* with IVM. Exposure of *B. malayi* to IVM was carried out after initial overnight incubation of mf in RPMI 1640. The mf were incubated for 72 h in volumes of 1 mL (250×10^3 mf/mL) in 24-well plates at 37 °C, 5% CO_2. RPMI 1640 containing 0, 0.1, 0.32, or 1.0 μM IVM was prepared by dilution of a 10 mM stock in DMSO. Control wells contained 0.1% DMSO. Media from each plate were collected and replaced with fresh media with or without drug every 24 h.

Protein Determinations. Protein determinations were performed with an EZQ protein quantitation kit (Invitrogen) with some modifications. Two-hundred-fifty microliters of either test medium or RPMI 1640 were spiked with 50 ng ovalbumin as an internal standard and concentrated to dryness in a speedvac. Samples were resuspended in 5 μL doubled distilled H_2O. The total sample was serially spotted in volumes of 2 μL on assay paper in the sampling plate. The assay paper was washed twice with MeOH for 5 min and then incubated with the EZQ reagent for 30 min. Final 2-min washes were performed three times with 10% MeOH, 7% acetic acid. Assay paper fluorescence was determined in a plate fluorimeter (FlexStation II; Molecular Devices) at 450 nm (excitation) and 610 nm (emission). Protein amounts were estimated by extrapolation from the standard curve and corrected according to the internal standard readings of RPMI 1640 alone.

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