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Endogenous morphine and parasitic helminthes

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RA

Summary

The current paper summarizes the findings on endogenous morphine isolated by HPLC and characterized by mass spectroscopy in *Schistosoma mansoni*, *Dracunculus medinensis* and *Ascaris suum*. Morphine-6-glucuronide (M6G) has also been found by HPLC and confirmed by mass spectroscopy in *Dracunculus medinensis* and *Ascaris suum*. In addition, a morphine like substance has been isolated from *Trichinella spiralis* by HPLC and mice infected with *Trichinella spiralis* show a naloxone reversible analgesia. We discuss in greater detail the tissue distribution, course of secretion, and sex differences of morphine in *Ascaris suum*. Finally, we explore the function of morphine as both an internal signaling molecule and its use in immune evasion in *Ascaris suum*.

key words: **morphine • helminthes • nitric oxide • immune evasion**

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BACKGROUND

Helminthes are multicellular parasitic worms that include both nematodes and trematodes infecting the digestive system. Helminthic infection is a major Public Health problem world wide with over two billion people currently infected with these worms [1]. However, a large percent of these infections are asymptomatic indicating that these worms are capable of down regulating the immune response against them. Despite being from rather diverse taxa, helminthes invoke a remarkably consistent immune response from their hosts [2]. They also typically evoke very little immune response despite their size and employ a variety of tactics for immune evasion [2,3]. A number of strategies of immune evasion have been documented including excretory-secretory antigens, antigenic cloaking, and immune suppression [4,5,3,6]. These parasites undergo complex life cycles assuming different forms in different hosts at various stages of their development and may employ a variety of immune evasion strategies during the course of their lives. The investigations of the immune modulating effects of opiates in our laboratories have lead us to examine several helminthic parasites for the presence of opiates and the effects of opiates in regard to immune evasion during these infections. The current review will examine endogenous morphine isolated by high performance liquid chromatography (HPLC) and characterized by mass spectroscopy (MS) in *Schistosoma mansoni*, *Dracunculus medinensis* and *Ascaris suum* [7,8]. Morphine-6-glucoronide (M6G) has likewise been found by HPLC and confirmed by mass spectroscopy in *Dracunculus medinensis* and *Ascaris suum* [7,9] and will be discussed. Additionally, a morphine like substance has been isolated from *Trichinella spiralis* by HPLC [10]. We will also describe in greater detail the tissue distribution, course of secretion, and sex differences of morphine in *Ascaris suum* [9]. Finally, we will discuss the function of morphine as both an internal signaling molecule and its use in immune evasion in *Ascaris suum.*

The greatest care was taken in all of these experiments to prevent contamination with exogenous morphine. To avoid morphine contamination of the biological samples tested, extraction experiments using internal or external standards were performed in separate rooms. The equipment was purged of any residual morphine by running HPLC blanks and non-morphine containing tissue runs between positive tissue runs. Finally, the fraction of the blank HPLC run corresponding to morphine was tested for morphine by mass spectroscopy and confirmed to be negative.

SCHISTOSOMA MANSONI

Schistosoma mansoni is a digenic trematode whose lifecycle includes two hosts, a definitive host (humans) where the parasite undergoes sexual reproduction, and a single intermediate snail host where there are a number of asexual reproductive stages. The adult male and female worms reside *in copula* in the portal veins above the intestines where the female lays hundreds of eggs daily that migrate into the intestines and are excreted with the host's feces [11]. Many infections are asymptomatic and there is little immune reaction against the adult stage but the migrating eggs often cause an inflammation that can become chronic [12]. Despite being multicellular, schistosomes can live in the blood vessels of the mammalian hosts for decades even in the face of an ongoing immune response by the host [13]. Both the adult and developmental stages are thought to be virtually invisible to the immune system [13].This is also true of the susceptible snail intermediate host [14]. However, the integument of the parasite is strongly antigenic and if the parasite dies then an acute immune response ensues [15].

A morphine like molecule in *Schistosoma mansoni* was first detected by Leung and colleagues some 10 years ago using HPLC [16] and radioimmune assay (RIA). More recently, we have isolated morphine from *Schistosoma mansoni* tissue using both HPLC and MS [7]. The mean concentration of morphine in five samples of *Schistosoma mansoni*, determined by extrapolation from the peak areas calculated for the external standards was 6.24 (2.83) ng/g wet weight. The M6G was absent from the elution. Analysis of HPLC fraction corresponding to the morphine and M6G fractions of the *Schistosoma mansoni* samples by regular Nano electrospray ionization double quadropole orthogonal acceleration time of flight mass spectroscopy (Q-TOF-MS) and ion fragmentation confirmed the presence of morphine and the apparent absence of M6G.

Endogenous material, corresponding to morphine in the original experiment of Leung and his colleagues, was found to mimic authentic morphine in its ability to induce immunocyte rounding and immobility, an action that is naloxone sensitive [16]. The effect of various experimental conditions on the production and release of morphine during incubation at 37° for 8 hours is depicted in Table 1 [16]. Worms incubated alone showed a drop in morphine like material to below detection and a concomitant appearance of a low concentration of morphine in the media. Coincubation with human polymorphonuclear leukocytes (PMN) increased the endogenous level of this material in adult worms, indicating the presence of a positive feedback loop. EDTA, a chelator of divalent cations, has a strong stimulating effect in the synthesis of morphine-like material by the worm as noted by higher levels of this material in its presence during incubation, especially in conjunction with PMN. The addition of KCl, a typical depolarizing stimulus did not cause an increase in morphine like material. However, incubation with EDTA, KCl, and PMN caused a reduction in the worm's morphine-like levels to levels noted with just PMN and a trace of morphine-like material in the incubation medium. Taken together, the results suggest that this parasite may utilize this immune down regulating molecule in its effort to escape host immunosurveillance as well as in inhibiting an immune response directed against it. The exact stimulus for morphine release by the worm may not be duplicated in this *in vitro* experiment but may involve interactions with the host organism.

TRICHINELLA SPIRALIS

Trichinella spiralis is an intestinal nematode whose emergent larvae migrate from the intestines and encyst in the host's muscle cells. These larvae are unique in their sequestering host's cells to act as "nurse cells" where they reside for many years [11]. While migration through the host's tissue does cause an immune response, most cases are asymptomatic and the larvae encased in a nurse cell is resistant to immune attack [17]. The presence of opiate like molecules has also

Table 1. The Presence of morphine-like substances in *Schistosoma mansoni* and Its incubation medium following various treatments.

Number of worms	PMN	EDTA (50 mM)	KCl (50 mM)	Morphine-like material in medium (pmol/100)	Morphine-like material in worm (pmol/100)
0	+			0	0
400				0.4	0
400			+	0	0
400		+		0	3.3
400		+	+	0	0.2
200	+			0	11.8
200	+		+	0	11.0
200	+	+		0	20.6
200	+	+	+	0.2	9.6

been examined in this parasitic nematode. Both morphine and codeine-like molecules were found to be present following HPLC separation and confirmed by RIA [10]. Like the material from *Schistosoma mansoni,* the endogenous material corresponding to morphine was found to mimic authentic morphine in its ability to induce *in vitro* immunocyte rounding and immobility. These effects were reversed by adding 10^{-6} M naloxone [10].

Infection with *Trichinella spiralis* in experimental animals induces alterations in nociceptive behavior which are blocked by the prototypic opiate antagonist naloxone [18]. Similar naloxone sensitive antinociception has been demonstrated in several other helminthes including *Schistosoma mansoni* [19], *Nippostrongylus brasiliensis* [20] as well as the intestinal protozoan, *Eimeria vermiformis* [21]. While opiate and opioid neuropeptide blood serum levels are increased by infection with both *Trichinella spiralis* and *Schistosoma mansoni,* it is not clear whether these substances are secreted directly by the parasites or produced endogenously as a reaction to parasitic infection [10]. There may, in fact, be a complex interaction between these two processes given the ability of morphine to induce the activation of opioid neuropeptides.

Dracunculus Medinensis

Dracunculus medinensis is a parasitic nematode that causes the disease known as dracunculiasis, and is often referred to as the guinea worm or fiery serpent. This parasite and the disease that it causes have been mentioned in ancient Greek, Roman, and Arabian texts. Some scholars believe that the "serpents" depicted in caducei (various medical symbols) are guinea worms symbolizing the common means of extracting the worms by wrapping them around a small stick, while others state the "fiery serpents" that plagued the Israelites were, in fact, *Dracunculus* [11]. The life cycle of *Dracunculus. medinensis* is unusual in many respects. Infection begins with the ingestion of a freshwater copepod called a Cyclops. Stomach acids dissolve the Cyclops and the free larvae penetrate the gut lining and migrate to subcutaneous tissues via the lymphatics. This migration takes about 43 days and once in the subcutaneous tissue the worms take about a year to mature into adults. After mating, the small males (1.2–2.9 cm) die and are absorbed into the larger female (60 cm in length but only about 2 mm in diameter). When the female is gravid with embryos, she migrates to the extremities (usually the feet and legs) which may likely be in contact with water and penetrates the skin. The uterus is extruded through the mouth releasing the embryos into water and the female dies. The female worm in the connective tissues of the limbs usually does not cause any noticeable pathological conditions. Most pathology is associated with infection occurring when the female dies after discharging her larvae [11].

Adult *Dracunculus medinensis* have been found to contain not only morphine but its active opiate alkaloid metabolite morphine-6-glucoronide (M6G) [7]. The mean concentration of morphine and M6G in five samples of *Dracunculus medinensis,* determined by extrapolation from the peak areas calculated for the external standards were 11.43 (4.57) and 5.72 (2.83) ng/g wet weight. Analysis of the HPLC fraction corresponding to the morphine and M6G fractions of the *Dracunculus medinensis* samples by regular Q-TOF-MS and ion fragmentation confirmed the presence of both morphine and M6G. It is quite possible that these immune suppressive substances may be used by *Dracunculus medinensis* for immune evasion, given the lack of immune response to the living nematode. Unfortunately, we were not able to perform experiments on excretion of morphine or M6G or experiments on immune response in these worms.

Ascaris Suum

Ascaris suum is perhaps the best known nematode to biology professors since it is so often used in basic biology course labs. It is one of the largest nematode measuring up to 50 cm in the case of females and 31 cm for males. This makes it easy to do studies on the worm's physiology and the distribution of substances in the various organs of its body. The adult worms live for years in the small intestine and eggs are passed in the feces. A single female can produce up to 200,000 eggs each day. The eggs become embryonated in about two weeks after passage and pigs are infected when they ingest such infective

Table 2. The distribution of morphine in male and female *Ascaris suum* tissue.

Nanograms/gram of wet tissue	Muscle	Lateral cord	Intestine	Uterus	Ovary	Testis
Female	252±32.68	10.58±1.43	81±10.71	147±12.83	53±4.61	
Male	180±23.47	8.6±2.64	72±6.71			38±7.45

eggs. After hatching in the small intestine, the juvenile penetrates the intestinal walls and enters the circulatory system, and eventually enters the lungs. In the lungs the juvenile worm leaves the circulatory system, entering the air passages of the lungs. Migrating up the air passages into the pharynx the juvenile worm is swallowed, and makes its way again to the small intestines where it grows into an adult worm. The adult worms subsist primarily on the contents of the gut and normally cause little immune reaction. The majority of infections (~85%) appear to be asymptomatic, in that there is no gross pathology seen. Most pathology is seen from the migrating larvae especially as they make their way through the lungs [11].

Ascaris suum contains the opiate alkaloid morphine as determined by HPLC coupled to electrochemical detection (ECD) and by gas chromatography/mass spectrometry [8]. The level of this material is 1168±278 ng/g worm wet weight. M6G was also detected in muscle tissue of both sexes of *Ascaris suum* by HPLC coupled with ECD and confirmed by MS analysis. The level of M6G in muscle tissue of the female worm is 167±28 ng/g. The level of M6G in muscle tissue of the male worm is 92±11 ng/g.

Ascaris suum of both sexes along with their incubation fluid were also analyzed for morphine concentrations by HPLC over a 5-day period [9]. The morphine level in the body was high in both male and female the first day of incubation. The morphine level went down precipitously in both males and females on the third day and descended to around 70 ng/g on the 4th day. The morphine level in the incubation medium was relatively high in both female and male the first day of incubation (138±31 ng/L, 126±27 ng/L). Morphine level decreased gradually over the 5-day period. There was no significant difference of morphine between male and female incubation medium. On day 4, morphine in the medium was at about the 70 ng/L level. The worms became less active as time progressed and mortality greatly increased after five days of incubation. The lack of a significant difference in secretion of morphine between the sexes was somewhat puzzling since there appeared to be a large amount of morphine surrounding the eggs of the female *in vitro* and eggs were, of course, continually passed in great abundance during incubation.

The Distribution of Morphine in Male and Female *Ascaris Suum* Tissue

The distribution of morphine in the body of the worm was examined using indirect immunofluorescence [8]. Immunoreactive morphine was found in subcuticle layers among collagenous structures, in fiber-like structures in the hypodermis, in the radial intercellular spaces between longitudinal contracting muscles underlying the skin, in the spaces encircling the inner big bodies of muscle cells, and in the nerve chords. Additionally, the animal's ova appear to be surrounded by this material in the extracellular space.

The worms were also dissected and morphine was quantified in different tissues by HPLC [9]. Morphine was found in muscle, intestine, testis, ovaries, and uterus. The level of morphine in different tissues was compared with an authentic morphine standard, extrapolated according to its peak area with Waters chromatographic software. The amount of morphine in various tissues from male and female *Ascaris suum* is indicated in the Table 2. The experiments were repeated 3 times for each tissue. The HPLC fraction corresponding to morphine was further analyzed by RIA. The results were in agreement with the HPLC data. The identity of the fractions was further confirmed by Q-TOF-MS analysis.

Opiate Receptors in *Ascaris Suum*

Tissues from *Ascaris suum* have also been examined for a human like μ opiate receptor. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was used to amplify a fragment of the coding region of the μ opiate receptor from *Ascaris suum* and also one from *Mytilus edulis* as a positive control. Using μ-specific primers, a transcript of the expected size for the μ receptor was isolated from *Mytilus edulis,* the positive control but not from *Ascaris suum.* This was not due to a lack of mRNA from *Ascaris suum,* because a 451-bp mRNA was amplified corresponding to G3PDH from the worm. Sequence analysis of the *Mytilus edulis* PCR product demonstrated that the μ receptor fragment exhibited 95% sequence identity with the human brain μ opiate receptor [8].

Real Time Determination of Nitric Oxide Release in *Ascaris Suum*

In real time, morphine and morphine-6-glucuronide (10^{-9} M) has been found to stimulate the release of nitric oxide (NO) from muscles [9]. Increasing concentrations of morphine and M6G (10^{-10} to 10^{-6} M) result in a dose-dependent increase in NO release with a maximal effect observed at 10^{-7} M (27 nM peak value). This increased peak was observed immediately after the addition of both alkaloids. Addition of 10^{-11} M of both compounds failed to stimulate a significant increase in NO release. Naloxone (10^{-7} M), a μ receptor antagonist, blocked ($P<0.005$) morphine-stimulated NO release from *Ascaris suum* muscle tissue. L-NAME (10^{-6} M), a NOS inhibitor, also blocked the NO stimulating activities of morphine. However, naloxone could not block M6G stimulated NO release by muscles, whereas CTOP (10^{-7} M) blocked its release. Interestingly, CTOP could not block morphine-stimulated release of NO from the muscle.

Discussion

In the past decade, several free living and parasitic invertebrates have been found to posses both opioid neuropeptides and the opiate alkaloid, morphine. The proopiomelanocortin gene has been isolated in *Schistosoma mansoni* [22] and opi-

oid peptide precursors including prodynorphin, proenkephlin, and proopiomelanocortin have also been isolated from invertebrates [23–27]. In addition to the parasitic helminthes that we have discussed, the presence of morphine-like substances has also been demonstrated in the mollusk, *Mytilus edulis* [28] and the leech *Theromyzon tessulatum* [29]. Opiates are known to be involved in the stress response in both vertebrates and invertebrates [30–32]. Morphine has also been long known to be immunosuppressive [33]. This ability of morphine to suppress the immune response of the host has lead to the hypothesis of the secretion of morphine by parasitic invertebrates for this purpose [8].

All of the parasitic helminthes that we have reviewed show little immune activation in their hosts despite being large long lived multicellular organisms. Interestingly enough, all of these parasites elicit a strong immune response if they are killed or die within the host. Obviously, something is being secreted by the living parasite to block this immune response. A number of strategies have been documented in each of these helminthes that allow these parasites to evade immune attack. Some of these involve secretion of immune modulators that block attack while not entirely suppressing the immune response. These excretory-secretory derived immune modulators include proteases [34], protease inhibitors [35], antioxidant proteins [36], and a homologue of human cytokine macrophage migration inhibitory factor [37]. Morphine may well be in the arsenal of these parasites, especially in the infective stages as they face immune attack but also as adults sequestered in tissues and the digestive system. It is possible that the opiate effects seen in experimental animals parasitized by some of these helminthes are caused by the release of opiates by the parasites

Another possible explanation for endogenous morphine in these organisms is its use as an internal signal molecule. Of particular interest is the release of NO in response to morphine and morphine-6-glucuronide by muscle tissue of *Ascaris suum.* This was in seeming contradiction to our earlier inability to isolate a μ opiate receptor messenger RNA by RT – PCR using a human μ primer [8]. Previous reports have also showed the presumed activity of NOS in *Ascaris suum* [38] and other nematodes [39–41]. FMRFamides have also been reported to cause NO release in *Ascaris suum* and to be responsible for a number of muscle responses [42–45].

There has been speculation that FMRFamides are evolutionarily related to opioid neuropetides and do in fact share some amino acid sequences [46,47]. FMRFamides have been shown to be antagonistic to some opiate actions in invertebrates [48,49] and to antagonize opiate activity in mice [49]. Opioid effects like decreased colonic bead expulsion in mice [50], and intrathecal-elicited, long lasting antinociception in rats [51] has been reported for FMRFamides. There is also evidence that FMRFamides may be involved in opiate tolerance and dependence [52–54]. FMRFamides have been shown to have their own distinct binding sites in mammals [55] and given their abundance in nematodes such as *Ascaris suum*, it is possible that morphine might be cross-reacting with an FMRFamide receptor. However, the lack of cross reactivity of FMRFamides with opioid sites casts doubt on this hypothesis [56].

Morphine causes nitric oxide release in human nerve and immune cells by stimulating intracellular calcium transients and the same mechanism has been demonstrated in invertebrates [57,58]. Morphine has been shown to be specific for a subset of μ receptors called μ_3 found in both vertebrate and invertebrate tissue [59,60]. Morphine but not opioid neuropeptides are able to bind to μ_3 and cause NO release. This effect is blocked by the general opiate antagonist, naloxone and the μ specific antagonist, CTOP in these tissues.

The pattern of nitric oxide release by morphine and morphine-6-glucuronide demonstrated in *Ascaris suum* is similar to that seen in *Mytilus edulis* nerve tissue [61]. Morphine and M6G stimulates *Mytilus edulis* pedal ganglia constitutive NO synthase (cNOS)-derived NO release at identical concentrations and to similar peak levels. However, naloxone only blocked the ability of morphine to stimulate cNOS-derived NO release and not that of M6G. CTOP blocked the ability of M6G to induce cNOS-derived NO release as well as that of morphine. It was also found that both opiate alkaloids displaced [3H]-dihydromorphine binding to the mu opiate receptor subtype in Mytilus. However, morphine exhibited a twofold higher affinity than M6G.

It quite possible that *Ascaris suum* possesses an opiate receptor that is specific for the morphine metabolite M6G rather than morphine itself. This would explain the lack of mRNA for the ĕ opiate receptor similar to human. It is interesting that nematodes also apparently lack several opiate neuropeptides that have been found in other invertebrates such as *Mytilus edulis.* It has been reported that immunoreactivity for β endorphin is absent in the nervous system of *Ascaris suum* [62] and that the gene coding for proopiomelanocortin is absent from the genome of *Caenorhabditis elegans* [63]. The finding that morphine and M6G cause nitric oxide release suggests that *Ascaris suum* does indeed use the morphine that it produces internally, probably as a means of dampening stress responses. We are presently investigating stress response in this animal and the role of opioid neuropeptides in *Ascaris suum.*

The internal release of nitric oxide in response to morphine and M6G does not discount the possibility that morphine also plays a role in immunosuppression of the host in parasitic helminthes. The relatively large amounts of morphine secreted into the incubation media of *Ascaris suum* give support to the idea that local secretion of morphine could be used by this intestinal parasite to avoid immune attack.The presence of a large amount of morphine on the cuticle and around the eggs in the ovaries also indicate a possible protective function of morphine. The fact that infection with *Ascaris suum* is usually asymptomatic indicates that these parasites elicit very little immune response.

In summary, several parasitic helminthes have been found to contain morphine or morphine like substance including *Schistosoma mansoni, Trichinella spiralis, Dracunculus medinensis* and *Ascaris suum. Dracunculus medinensis* and *Ascaris suum* have also been found to contain morphine-6-glucuronide. Experimental animals infected with parasitic helminthes exhibit antinociceptive behavior which is reversed by injection with naloxone. Morphine from parasitic helminthes cause immune cell inactivation and exposure to immune cells *in vitro* may induce the production of morphine by these worms. Morphine shows a wide tissue distribution in *Ascaris suum*, being present in the cuticle, nervous tissue, muscle tissue,

intestinal tissue and reproductive tissue. While a human like μ receptor is not apparent in *Ascaris suum*, morphine and M6G do cause NO release in *Ascaris suum* tissue. This indicates that morphine or M6G may be used for internal signaling. However, the rather large secretion of morphine during incubation by *Ascaris suum* and the effect of morphine on host immune cells also indicate a possible immune suppressive function for morphine in these parasites.

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