Ascaris lumbricoides: An Overview of Therapeutic Targets

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Abstract: *A. lumbricoides* is the largest of the common nematode parasites of man and has been associated with intestinal pathology, respiratory symptoms and malnutrition in children from endemic areas. Current anthelmintic treatments have proven to be safe. However, a reduced efficacy of single dose drugs has been reported. In veterinary practice, anthelmintic drug resistance is an irreversible problem. Thus, research and development of sensitive tools for early detection of drug resistance as well as new anthelmintic approaches are urgently needed. In this review, we summarized data providing information about current drug therapy against *A. lumbricoides* and other intestinal helminths, new drugs in experimental trials, future drugs perspectives and the identification of immunogenic parasite molecules that may be suitable vaccine targets. In addition to the WHO recommended drugs (albendazole, mebendazole, levamisole, and pyrantel pamoate), new anthelmintic alternatives such as tribendimidine and Nitazoxanide have proved to be safe and effective against *A. lumbricoides* and other soil-transmitted helminthiases in human trials. Also, some new drugs for veterinary use, monepantel and cyclooctadepsipeptides (e.g., PF1022A), will probably expand future drug spectrum for human treatments. The development of genomic technology has provided a great amount of available nematode DNA sequences, coupled with new gene function data that may lead to the identification of new drug targets through efficient mining of nematode genomic databases. On the other hand, the identification of nematode antigens involved in different parasite vital functions as well as immunomodulatory molecules in animals and humans may contribute to future studies of new therapeutic approaches.

Keywords: Anthelmintic, *Ascaris lumbricoides*, cyclooctadepsipeptide, nematode, nitazoxanide, tribendimidine.

INTRODUCTION

Ascariasis caused by infection with the nematode *Ascaris lumbricoides* is one of the soil transmitted helminthiasis (STH) that head the list of the so called "Neglected Diseases" [1]. *A. lumbricoides* is the largest of the common nematode parasites of man. Approximately 1300 million of individuals are infected with this parasite being its morbidity about 120- 220 million (8-15%) of the total number of infected people [2]. It has been estimated that a female worm has the potential to produce over 200000 eggs per day. Eggs are passed in the faeces in the unembryonated state. Infective eggs survival is variable up to a period of 15 years. Most of the eggs are thought to be destroyed soon after passage although many will embryonate to produce second stage larvae under adequate environmental conditions. Humans are infected by ingestion of embryonated eggs through fecal contamination. *A. lumbricoides* eggs can be found elsewhere: adhering to utensils, furniture, money, fruits, vegetables, doors, hands and fingers particularly in places in which conditions such as poor quality of housing and low sanitation are present. When eggs hatch in the duodenum the larvae pass through liver *via* portal circulation and migrate to the heart and lungs. Then larvae develop, molt and move up bronchi to trachea and pharynx where they are swallowed passing through the esophagus and stomach to the small intestine where they mature into adult worms [3].

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It has been reported that larval migration can lead to the onset of respiratory symptoms which can include asthma, cough, and wheezing [4]. The possible influence of *A. lumbricoides* infection on the development and worsening of concomitant respiratory infections has not been studied nevertheless would be of great importance since acute respiratory infections (IRA) are one of the major causes of dead in young children in developing countries [5]. Infection by *A. lumbricoides* has been also associated to intestinal pathology and particularly to malnutrition affecting growth, fat and vitamin A absorption [6]. In addition it has recently become clear that helminths parasites are important not only because of the pathogenic effects directly attributable to them but because they interact with the immune system in many ways which may impair the response to other infectious agents [7]. For example, co infection with *A. lumbricoides* and other common helminths has been associated to the worsening of tuberculosis [8] and malaria [9]. Thus the control of ascariasis may be an important goal of public health programs in tropical developing countries.

Intensity of *A. lumbricoides* infection peaks in age ranging from 5 to 15 years old and declines in adults indicating the presence of acquired immunity within age [10]. Exposure to repeated infections during early life may induce some degree of protection [2]. However studies in human populations following chemotherapy have shown moderate rates of re-infection in relative shorts periods (12-18 months) [11-13] indicating that acquire resistance generated by past infections is only partial in many individuals. In addition there is evidence of predisposition to *A. lumbricoides* infection among human populations [14]. Thus people with heavy or light worm loads either as a group or as individuals tend to re-

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acquire respectively heavy or light infections. The number of worms within a host population is not normally distributed but follows an aggregated or over-dispersed frequency distribution [15]. This means that in a given population most hosts will harbor few or no worms while a small proportion of them will carry heavy infections. Heavily infected people are more at risk from morbidity and also act as significant contributors of potentially infected stages in the environment [15].

Studies carried out in murine model systems have emphasized the importance of genetically determined variability in resistance to gastrointestinal (GI) nematodes [16]. In humans there are a few studies attempting to elucidate the genetic basis of predisposition against these parasites. William-Blangero *et al.* [17] carried out a genetic epidemiologic analysis of *A. lumbricoides* infection in the Jirel population of eastern Nepal. A total of 1,261 individuals belonging to a single pedigree were assessed for intensity of *Ascaris* infection at two time points. Following an initial assessment in which all individuals were treated with albendazole, a follow-up examination was performed one year later to evaluate reinfection patterns. For all traits, variance component analysis of the familial data provided unequivocal evidence for a strong genetic component accounting for between 30% and 50% of the variation in worm burden. Interestedly, shared environmental (i.e., common household) effects in this study only accounted for between 3% and 13% of the total phenotypic variance. Further studies in the same population resulted in the localization of two genes: one on chromosome 1 and another on chromosome 13 with significant effects on susceptibility to *A. lumbricoides* infection [18]. In this study significant linkage were found in two regions: 13q32-q34 and 1p32. The 13q32-q34 region has been linked to the control of antibody responses including the elevation of total IgE levels that is a hallmark of the immune response against these parasites [18]. Ramsay *et al.* [19] examined associations between two coding polymorphisms at positions 16 (ArgGly) and 27 (GlnGlu) in the B2 adrenoreceptor gene (ADBR2) on 5q31-q33 with the intensity of infection with *A. lumbricoides* among Venezuelan rural children. It was found that those children with the Gly16 or Glu27 alleles which are in linkage disequilibrium exhibited significantly lower burdens than children homozygous for Arg16 or GLn27. Studies carried out in *A. lumbricoides* parasitized children of an endemic rural area of Shanghai, China have shown that a 3' variant of the UTR variant of Stat6 located in the chromosome 12 was the principal predictor of the intensity of the infection [20]. The Stat6 molecule is involved in specific signaling of Th2 type cytokines (IL-13 and IL-4) that are known to be stimulated during helminthic infections [21]. Thus susceptibility against *A. lumbricoides* in humans seems to be influenced by particular genotypes among different human populations.

Anti-helminthic drugs against *A. lumbricoides* have found to be safe and effective. Single oral doses of Albendazole, Mebendazole and pyrantel pamoate have demonstrated high cure rates against this parasite [1]. However rates of reinfection after chemotherapy have shown to be high [11,12,13,15] probably due to the lack of sanitary facilities frequently observed in rural areas of developing countries in which most of these studies were carried out. Thus, many repeated doses of anti-helminthic drugs would be required to low the prevalence and intensity in endemic areas in which improvement of socioeconomic conditions are far from reality. This would lead to different problems such as acquisition of drug resistance [2]. On the other hand drug waste may pollute the environment particularly in rural areas where there are no adequate systems for sewage disposal and massive anti-parasite programs may overlap with continuous use of veterinary anti -helminthic treatments. It is possible that the development of effective anti-helminthic vaccines would contribute to a solution for these problems. However the development of such vaccines has been slow, probably due to the complexity of life cycles [3] and the ability of these parasites to evade and modulate the immune response of the host. [7]. In addition attempts to produce effective vaccines against other helminthes parasite have not made a major impact on medical or veterinary practice. However a promising vaccine to human hookworms is currently being developing [22]. Also studies using recombinant vaccines to *Ascaris suum* are of particular interest because they may be a suitable model to develop an effective vaccine against *A. lumbricoides* [23].

The focus of this review is to summarize different data providing information about current drug therapy against *A. lumbricoides* and other intestinal helminths, future drugs perspectives and the role of different molecules involved in host - parasite associations in animal and human models that may contribute to future studies for new therapeutic approaches for the control of human ascariasis as well as other intestinal helminths.

PHARMACOLOGICAL ASPECTS OF CURRENT DRUGS

Four anthelmintics are currently recommended by the World Health Organization for treatment and control of Soil Transmitted Helminthiasis (STH): albendazole, mebendazole, levamisole, and pyrantel pamoate. The four of them has been successfully used in the control of *A. lumbricoides* [1].

It is well known that target sites of these drugs are mainly proteins: ion channels, enzymes, structural proteins and transport molecules. Drugs acting on ion channels have typically a rapid effect. In contrast drugs directed to more "biochemical" target sites often act more slowly [24]. Details of the pharmacology of classical anthelmintic drugs have extensively been reviewed and therefore these aspects will only briefly be covered here.

ANTIMICROTUBULE AGENTS

Benzimidazole-based compounds are the most widely used anthelmintics until now. The therapeutic effect of these drugs lies on their ability to bind with high affinity and in a pseudo-irreversible fashion to the β-subunit of the tubulin protein, disrupting microtubules structure and functions [25]. The loss of microtubules leads to subsequent death of the organism. The selectivity shown by benzimidazoles has been verified in colchicine-assays, testing the *in vitro* polymerization of tubulin in mammals and nematodes. In most cases, this inhibition was more pronounced in nematode tubulin than the detected from mammalian tissues, indicating that benzimidazole group of drugs interacts selectively with

nematode tubulin [26]. Synthetic benzimidazole peptides have also been tested for antimicrobial, cytotoxic and anthelmintic effects revealing a good level of activity when compared to the standard drug (reviewed in [27]).

DRUGS ACTING ON THE NEUROMUSCULAR SYSTEM

Two of the three major types of anthelmintic, the avermectins and the nicotinic agonists, exert their therapeutic effect by an action on ligand-gated membrane ion-channels of nematodes. The avermectins such as ivermectin, open glutamate-gated chloride channels only in invertebrate preparations [28] whereas nicotinic anthelmintics like levamisole, selectively gate nematode nicotinic acetylcholine receptors [29,30]. The surface of somatic muscle cells on nematodes exhibit nicotinic acetylcholine receptors (nAchR) that can be opened by nicotinic - anthelmintic drugs [31,32]. Nicotinic Acetylcholine Receptors (nAChR) are receptor-operated cation channel, composed of a pentameric structure built up from different subunits. Each α-chain of the channel contains a binding site for acetylcholine. The subunit composition and stoichiometry of this receptor can vary between different subtypes resulting in a functional diversity of nAChR [31,33]. Electrophysiological characterization of the native nAChRs have indicated that 3 distinct pharmacological nAChR subtypes are present on *Ascaris* muscle cells, with different agonist and antagonist sensitivities: an L-subtype most sensitive to the agonists levamisole and pyrantel, an N-subtype most sensitive to nicotine, oxantel and methyridine, and a B-subtype most sensitive to bephenium [34]. Binding of these compounds to the recognition site of the excitatory receptor produces depolarization and spastic paralysis of the nematode muscle that can result in parasite expulsion. The selective toxicity of these compounds appears to be based on the unique properties of the nematode nAChR that are pharmacologically distinct to those of the higher animal [34]. On the other hand, macrocyclic lactone such as avermectins (e.g., ivermectin and doramectin) and milbemycins (e.g., moxidectin) which lack the glycosidic substitution are extremely potent anti-nematode drugs [28]. These drugs are widely used to treat infections in animals but are also the drugs of choice to control human onchocerciasis [35]. A study on the effects of ivermectin on pharyngeal pumping of the nematode *Haemonchus contortus* have shown that this drug produce a flaccid paralysis of the somatic worm musculature and inhibit feeding of the parasite by blocking pharyngeal pumping. The latter effect is exhibited at chemotherapeutically relevant levels, and it has been suggested that disruption of ingestive activity and worm starvation is the real nematicidal action of these compounds [36]. The first genes encoding GluCl subunits, *glc*-1 (GluCl α-1) and *glc*-2 (GluCl β), were identified in *C. elegans* by functional expression in *Xenopus* oocytes [37]. GluCl α-1 and GluCl β associate in oocytes to form a heteromeric glutamate- gate chloride channel that is sensitive to ivermectin. Ivermectin appears to act as an agonist of glutamate by increasing the open times of the receptor [38]. At low concentrations it potentiates the effect of the natural transmitter and at higher levels opens the channel directly. The binding of ivermectin results in irreversible chloride ion currents followed by hyperpolarization of the cell membrane and muscle paralysis. The selective effect of these compounds is explained by their action on the distinct GluCl that are unique to invertebrates but absent in the vertebrate host [38]. Piperazine, acts as a GABA agonist by opening GABA-gated chloride channels present on nematode somatic muscle cells. Binding to these receptors induces an increase in chloride permeability of the muscle cell membrane that eventually results in a relaxation of the body musculature and flaccid worm paralysis [39].

EFFICACY OF CURRENT ANTHELMINTICS IN *A. LUMBRICOIDES* INFECTION

A key aspect in the control of human intestinal nematode infections is the availability of studies assessing the efficacy for each drug in distinct populations of nematodes. Although this information would be crucial for guiding national STH control programs, significant methodological deficiencies regarding study design, sample size, diagnostic and post-treatment control methods have indicated the need for more adequate studies that would help to the development of therapeutic control of intestinal nematode infections [40]. Nevertheless a number of studies have compared the efficacy of various anthelminthic drugs in communities parasitized with *A. lumbricoides* and other STH. A study carried out by Hadju *et al.* [41] in Indonesia found that both albendazole and pyrantel were efficient in lowering significantly ($p<0.005$) the prevalence in parasitized school children compared to the placebo group after two doses yearly. On the other hand other studies have reported highly effective single dose for *A. lumbricoides* using albendazole or mebendazole [42,43]. Also levimazole [44] and ivermectin [45] have been shown to be effective in reducing the prevalence of *A. lumbricoides*. Co-infection with other helminths such as *T. trichiura, S. stercoralis* or hookworm is an important factor to take in account for proper anthelminthic choice in a given population. For example unlike pyrantel or mebendazole that are suitable drugs against *A. lumbricoides* only treatment with albendazole is effective against both adult and larval stages of hookworm that are very frequent in poly parasitized populations [42].

ANTHELMINTIC RESISTANCE

Drug resistance is defined as a state of insensitivity or decreased sensitivity to the effect of a determined drug concentration that normally cause growth inhibition or cell dead. Genetic modifications that confer resistance may reflect different biochemical modifications such as cellular changes that affect the capacity of the drug to accumulate into the cell, alteration of enzymatic systems and/or alteration of cellular receptors. In veterinary practice, frequent treatment of closed populations has led to a serious problem of anthelminthic drug resistance which is now largely irreversible [46]. Reduced efficacy of single dose drugs against nematodes of humans [40,43,47] should be taken as early warnings to tackle the issue in due time. Anthelminthic resistance is a genetic modification mediated by an increase in the frequency of the expression of a hereditable character that confers to a number of parasites of a given population the capacity to survive to the pharmacological effect of recommended therapeutic doses of an anthelminthic drug. Distinct factors affect the development of drug resistance on animal and hu-

man populations. Some of them are the relative frequency of resistance alleles present in the initial untreated population, the number of genes involved in resistance and their dominance/recessiveness, the degree of the drug pressure, the availability of other drugs for control programs, the number of treatments per year (been usually more often in veterinary use), under dosing [48].

In the case of cholinergic agonists it has been demonstrated that resistance is produced by a change in the properties of nicotinic receptors leading to a decrease in the sensitivity of the receptor to the drug. Also a decrease in the number of active receptors may occur. There is evidence that multiple types of nicotinic receptors are present in several nematodes and numerous genes are required to form these multimeric proteins. Resistance to these drugs is not necessarily the result of a single mutation but may be polygenic in nature. Also, the mutations resulting in resistance may vary between different species or between resistant isolates of the same species [49].

On the other hand it has been demonstrated that mutations in multiple GluCl subunit genes are required for high-level of macrolitic lactone resistance in the free living nematode *C. elegans* [50]. Even though parasitic nematodes differ in their complement of channel subunit genes from C. *elegans*, a few genes, including avr-14, are widely conserved. In according, a polymorphism in an avr-14, which makes the subunit less sensitive to ivermectin and glutamate, has been identified in *Cooperia oncophora* [51] and polymorphisms in several subunits have been reported from resistant isolates of *H. contortus* [52] suggesting that resistance to ivermectin may be polygenic.

Resistance to benzimidazoles in many parasitic nematode species from animals is usually due to a single nucleotide polymorphism (SNP) which causes an amino acid substitution from phenylalanine (Phe, TTC) to tyrosine (Tyr, TAC) in parasite β-tubulin at codon 200. A similar SNP at codon 167 (Phe167Tyr) or a glutamate to alanine change at codon 198 (Glu198Ala) can also occasionally be associated with benzimidazole resistance [53-55]. In humans, recent studies using Pyrosequencing assays for detecting the TTC or TAC SNP at codon 200 in β-tubulin in *A. lumbricoides* and *T. trichiura* were developed. The assays were applied to adult worms from a benzimidazole-naive population in Kenya. Following this, these assays were applied to individual worms and pooled eggs from people in East Africa (Uganda and Zanzibar) and Central America (Panama) where mass anthelmintic drug programs had been implemented. All *A. lumbricoides* samples were TTC. However, 0.4% homozygous TAC/TAC were found in *T. trichiura* worms from non-treated people in Kenya, and 63% of T. trichiura egg pools from treated people in Panama contained only TAC. The authors conclude that even though the codon 200 TAC SNP was not found in any of the *A. lumbricoides* samples analyzed, a rapid genotyping assay has been developed that can be used to examine larger populations of this parasite and to monitor for possible benzimidazole resistance development. The occurrence of the TAC SNP at codon 200 of β-tubulin in *T. trichiura* may explain why benzimidazole anthelmintics are not always highly effective against this species of STH. These assays will be useful in assessing appropriate treatment in areas of high *T. trichiura* prevalence that frequently co-infects with *A. lumbricoides* and in monitoring for possible resistance development in these STH [56].

Most of the research on anthelmintic resistance has been based on the "candidate gene studies" that may have limited utility since the data generated do not explain the whole genetics of resistance, how many loci are involved, their dominance relationships or differences in the genetic basis of resistance between isolates/species. The development of new techniques may be important for future studies.

In human populations at the community level, the fecal egg count reduction test (FECRT) is being using to test the possibility of drug resistance. Cut-off values of 50 and 70% have been suggested for *T. Trichuria* and *A. lumbricoides* respectively [57]. Given the low sensitivity of the FECRT, more sensitive assays are urgently needed. On the other hand, as mentioned above, a reduction in the efficacy of current anthelminthic treatments is also considered a sign of emergence of drug resistance in a given geographically area [40]. Regardless its scarce, the available evidence in anthelminthic resistance in human population indicated the need of more care in routine use of the anthelminthic also continuous drug efficacy surveillance must be a strong general recommendation. Anthelminthic control programs lying exclusively on drugs must be avoided to maintain the efficacy of the currently available drugs. In addition further research to provide efficient alternative therapeutics such as new drugs or the development of suitable vaccines seems to be urgent.

NEW DRUGS IN VIEW

Currently the most successful drug in human trials for new anti- intestinal helminthic approaches is tribendimidine which consists in a symmetrical diamidine derivative of amidantel [58]. This drug has been developed by the Chinese National Institute of Parasitic Diseases during the 1980s [58]. Recent experimental work has demonstrated that tribendimidine is an L-subtype nAChR agonist of the same family as levamisole and pyrantel sharing their same mechanism of action [59]. Laboratory and clinical investigations demonstrate that this drug is safe and has a broad spectrum of single-dose activity against parasitic nematode infections in humans, including *A. lumbricoides*, hookworms and *Strongyloides stercoralis* with reported cure rates of 92-96%, 52-90%, and 55% respectively [60], thus being an important new drug with broad anti-parasite activity. It was approved for human use by the China State Food and Drug Administration in 2004 and is currently undergoing clinical testing in China [61].

Another drug that may be suitable particularly in rural areas in which *A. lumbricoides* frequently co-infects with protozoa intestinal parasites is Nitazoxanide (NTZ). This compound was synthesized based on the structure of niclosamide. *In vitro* studies have demonstrated activity against a broad range of parasites as well as some bacteria. Three controlled trials demonstrated efficacy in cryptosporidiosis, however, the efficacy in advanced AIDS patients (CD4 cell counts = 50) at approved doses was limited. Trials have also demonstrated efficacy comparable to metronidazole in giardiasis with fewer side effects (reviewed [62]).

Also, NTZ has shown to be effective against *A. lumbricoides*, *T. trichiura*, and *Hymenolepis nana* [63] although, some patients require repeated dosing. A more recent study was conducted in children, teenagers and adults in a rural community in Colima, Mexico to examine the prevalence and intensity of *A. lumbricoides* infection and to evaluate the parasitological and clinical efficacy of NTZ [64]. Two hundred and eighty children, teenagers and adults participated in this study. Parasitological diagnosis from faeces was confirmed by three consecutive stool samples using the floatation concentration Faust method. Egg counts were performed as described by the Kato-Katz technique before and after treatment. It was found that (38%) of the individuals that participated in the study were diagnosed as harboring intestinal parasites, and 86 of them (81%) were infected with *A. lumbricoides*. All patients with ascariasis infections underwent a complete physical examination before and after NTZ treatment. NTZ resolved 88% of the ascariasis cases, with an 89% clinical efficacy, and there was a 97.5% reduction in the levels of morbidity [64].

Most recently, the amino-acetonitrile derivatives (AADs) were found to express anthelmintic activity in animals. The AADs are a class of low molecular mass compounds that are easily accessible by alkylation of phenols with chloroacetone, Strecker reaction and acylation of the amine with aroyl chlorides. They exhibit their anthelminthic action involving a nematode-specific clade of acetylcholine receptor subunits. The AADs are well tolerated and of low toxicity to mammals, and overcome existing resistances to the currently available anthelmintics [65]. Among ADDs, one of them, monepantel (AAD 1566), has been proposed for drug candidate after *in vitro* assays and efficacy and tolerability studies in rodents, sheep, and cattle [66]. Recently nine dose confirmation studies were conducted in Australia, New Zealand and Switzerland to confirm the minimum therapeutic oral dose of monepantel to control fourth stage (L4) gastrointestinal nematode larvae in sheep (target species were *Haemonchus contortus, Teladorsagia (Ostertagia) circumcincta, Teladorsagia trifurcata, Trichostrongylus axei, Trichostrongylus colubriformis, Trichostrongylus vitrinus, Cooperia curticei, Cooperia oncophora, Nematodirus battus, Nematodirus filicollis, Nematodirus spathiger, Chabertia ovina* and *Oesophagostomum venulosum*). In each study, sheep infected with a defined selection of the target nematodes were treated with 2.5mg monepantel/kg live weight. Following euthanasia and worm counting, efficacy was calculated against worm counts from untreated control groups. The results demonstrate high (95<100%) efficacy of monepantel when administered orally to sheep at 2.5 mg/kg for most species tested. Efficacy was demonstrated against L4 stages of nematodes known to be resistant to either benzimidazole and/or levamisole anthelmintic [67]. The same results were obtained results in a similar study conducted by other group of researchers conducted on 18 farms located throughout the North and South Islands of New Zealand [68]. The broad-spectrum activity of monepantel against L4 larvae of common gastro-intestinal nematodes in sheep and its favorable safety profile represents a significant advance in the treatment of parasitic gastro-enteritis in this animal species. Due to the excellent tolerability of the AADs in ruminants [69], the class may offer an alternative anthelmintic for human. Nevertheless it has been found that monepantel sulfone which is an active metabolite is the main chemical entity present in sheep blood after monepantel administration [70] thus its pharmacokinetic properties are of primary importance for the interpretation of future residue and efficacy studies. It remains subject to further evaluation if monepantel will become a commercially available anthelmintic product.

Another potent anthelminthic that has been used successfully in sheep is Paraherquamide (PHQ). This drug is an oxindole alkaloid originally isolated from cultures of *Penicillium paraherquii* [71]. The anthelmintic activity of PHQ was initially detected in the jird / *Trichostrongylus colubriformis* model and was found to be less potent than the macrocyclic lactones, but is more potent than the benzimidazoles, imidazothiazoles and tetrahydropyrimidines [72]. Results obtained from muscle tension experiments and measurements of muscle cell potentials have supported the hypothesis that PHQ class of anthelmintics acts as nicotinic ACh antagonists in nematodes [73]. It has been proposed that these compounds represent the first class of receptor antagonists with useful anthelmintic activity, since other anthelmintics acting on ion channels are all agonists. The use of this anthelmintic in sheep has been effective and safe [74]; nevertheless, there is yet limited application for PHQ on animal and human, since investigated compounds showed toxicity and lethality in mice, horse and dog including depression, ataxia and protrusion of the nictitating membrane at doses ranging from 0.5 to 2 mg/kg [75]. These effects can be explained because PHQ exerts its nematicidal action by inducing paralysis through blockade of cholinergic neuromuscular transmission [75].

It has been reported that the cyclooctadepsipeptide molecule, PF1022A has anthelminthic properties. PF1022A is a natural compound from the fungus *Mycelia sterilia* that belongs to the micro flora of the leaves of the *Camellia japonica* [76]. It contains 4 N-Methyl-L-leucines, 2 D-lactic acids and 2-D-phenyllactic acids arranged as a cyclic octadepsipeptide with an alternating L-D-L-configuration. In addition, emodepside a semi-synthetic derivative of PF1022A has shown anthelminthic activity [76]. The effects of cyclooctadepsipeptides have been studied on benzimidazole-, levamisole- and ivermectin-resistant populations of *H. contortus* in sheep as well as an ivermectin-resistant *C. oncophora* population in cattle. For these purposes experimentally infected sheep and cattle were used. Animals were treated orally, subcutaneously, or intravenously with cyclooctadepsipeptides. The anthelmintic effects were assessed by means of fecal egg count reductions and/or worm count reductions. Both, PF1022A and emodepside were found to be fully effective against these parasite populations [77]. It has been proposed that emodepside exerts its action on nematodes *via* a latrophilin-like receptor which may have an important regulatory function on pharyngeal pumping [78,79]. Electrophysiological studies revealed that emodepside inhibits pharyngeal pumping of the nematodes in a concentration dependent way. The mode of action is not yet known in detail. The activity is synergistically enhanced by piperazine, which indicates a possible involvement of the GABA system in the mechanism of action [78]. Future studies may elucidate the important role of PF1022 family and

related cyclodepsipeptides as promising drug candidates for animals and humans.

EXPANDING DRUG TARGETS

A better understanding of parasite genomes, host-parasite relationships and the molecular biology of parasites themselves will enable the rational development of diagnostic tests and/or safe anti-parasitic compounds. The application of recombinant DNA techniques for cloning of parasite receptors, ion channels and protein involved in signal-transduction available in the last years can be expected to lead to rapid progress in this field. The development of genomic technology has enabled *in silico* selection of drug targets in major human pathogens using rational target-based approaches [80]. In the case of nematode the use of a related model organism as a proxy for missing functional genomic data and applying multiple layers of subtractive filters based on comparative sequence analysis leaded to the possibility of validating a pool of targets to facilitate their entry into drug discovery programs [81]. This methodology was tested successfully in parasitic nematodes and has been endorsed by the World Health Organization as a promising approach to identify new anthelminthic drug targets [81]. Expressed sequence tag projects have currently produced over 400 000 partial gene sequences from more than 30 nematode species and the full genomic sequences of selected nematodes have been determined. In addition, functional analyses in the model nematode *C. elegans* have addressed the role of almost all genes predicted by the genome sequence [82]. This great amount of available nematode DNA sequences, coupled with new gene function data, provide an unprecedented opportunity to identify pre-validated drug targets through efficient mining of nematode genomic databases. For example, the genomic of parasitic nematode had been analyzed by Parkinson *et al.* [83] using 265,494 expressed-sequence tag sequences, corresponding to 93,645 putative genes, from 30 species, including 28 parasites. From 35% to 70% of each species' genes had significant similarity to proteins from the model nematode *C. elegans*. More than half of the putative genes were unique to the phylum, and 23% were unique to the species from which they were derived. More than 2,600 different known protein domains were identified, some of which had differential abundances between major taxonomic groups of nematodes. Among them and as particular interest for drug and vaccine targets 4,228 nematode-specific protein families from nematode-restricted genes were found. More precise studies using genomic sequence of *B. malayi* and the free -living nematode *C. elegans* as a surrogate have provided a genome - wide search for new drug targets [84]. Sequence comparisons between the two genomes enabled the mapping of *C. elegans* orthologous to *B. malayi* genes. The use of these orthology mappings and by incorporating the extensive and functional genomic data, including genome-wide RNA interference (RNAi) screens for *C. elegans* have allowed the identification of potentially essential genes in *B. malayi*. By virtue of an adequate selection procedure, the potential B. *malayi* drug targets highlight components of key processes in nematode biology such as central metabolism, molting and regulation of gene expression. Among these targets of great importance was the fact that microtubules and nervous systems appear to be the main chemotherapeutic targets in helminths. More recently Yin *et al*. [85] investigated phylum-specific molecular characteristics in Nematode by exploring over 214,000 polypeptides from 32 species including 27 parasites. Over 50,000 nematode protein families were identified based on primary sequence, including approximately 10% with members from at least three different species. Features of these protein families were revealed through extrapolation of essential functions from observed RNAi phenotypes in *C. elegans*, bioinformatics-based functional annotations, identification of distant homology based on protein folds, and prediction of expression at accessible nematode surfaces. Of great importance, a group of nematode-restricted sequence features in energy-generating electron transfer complexes was identified. This phyla-specific energy generation mechanism which is significantly distinct from oxidative phosphorylation pathways used by mammalian hosts [86], offers a prime target for the development of next generation parasite control strategies with potentially high specificity and minimal toxicity. Also, comparative analysis of key ANTs (adenine nucleotide translocators) and their genes in nematodes and other organisms are currently being studying in order to predict the potential of ANTs and associated molecules as possible drug targets [87]. ANTs belong to the mitochondrial carrier family (MCF). ATP production and consumption are tightly linked to ANTs, the kinetics of which have been proposed to play a key regulatory role in mitochondrial oxidative phosphorylation [88] and also in apoptosis [89]. Recently A full-length cDNA (Tv-ant-1) encoding an adenine nucleotide translocator (ANT or ADP/ATP translocase) (Tv-ANT-1) has been isolated from *T. vitrinus* [90]. Comparison with selected sequences from the free-living nematode *C. elegans*, cattle and human showed that Tv-ANT-1 is relatively conserved. In the same study, using transcriptional analysis by reverse transcription polymerase chain reaction (RT-PCR), was shown that Tv-ant-1 was transcribed in all developmental stages of *T. vitrinus*, including the first- to fourth- stage larvae [L(1)-L(4)] as well as female and male adults [90]. Further studies are needed to elucidate the expression of ANTs and its function in gastrointestinal nematodes in which may play an important role.

Energy routes have also been explored for anthelminthic therapeutic. Helminths have exploited a variety of energy transducing systems in their adaptation to the peculiar habitats in their hosts. Therefore, differences in energy metabolisms between the host and helminths are attractive therapeutic targets of helminthiasis. NADH-fumarate reductase is part of a unique respiratory system in parasitic helminths and is the terminal step of the phospho*enol*pyruvate carboxykinase-succinate pathway, which is found in many anaerobic organisms. The composition and linear sequential order of the respiratory components of NADH-fumarate reductase have been elucidated in the mitochondria from the parasitic nematode, *Ascaris suum*. Thus, electrons from NADH are accepted by rhodoquinone through complex I (NADH-rhodoquinone oxidoreductase) and then transferred to fumarate through complex II (rhodoquinol-fumarate reductase). This anaerobic electron transport couples site I phosphorylation in complex I by translocating protons across the inner mitochondrial membrane, providing ATP even in the absence of oxygen (reviewed in [91]). It has been re-

ported that nafuredin which is a delta-lactonic antibiotic, inhibits NADH-fumarate reductase (complexes I + II) activity in helminth mitochondria, at nM order. It competes for the quinone-binding site in complex I and shows high selective toxicity to the helminth enzyme [92]. Moreover, nafuredin has shown to exert anthelmintic activity against *H. contortus* in *in vivo* trials with sheep [92]. Thus, mitochondrial complex I is a promising target for chemotherapy, and nafuredin may be a potential anthelmintic isolated from microorganisms. Further studies demonstrated that nafuredin is easily converted to nafuredin-gamma by weak alkaline treatment. The structure of nafuredin-gamma was elucidated as a gamma-lactone form of nafuredin with keto-enol tautomerism. Nafuredin-gamma shows similar complex I inhibitory activity as nafuredin, and it also possesses anthelmintic activity *in vivo* [93].

Other sources of anthelmintics are the neuropeptides which have diverse roles in the function and development of the nervous system. Many organisms, including mammals, use short peptides as neurotransmitters. The family of FMRFamide (Phe-Met-Arg-Phe-NH2)-like neuropeptides, which all share an -RFamide sequence at their C-termini, has been shown to have diverse functions, including neuromodulation and stimulation or inhibition of muscle contraction (reviewed in [94]). It has been proposed that the employment of nematode neuropeptide receptors in mechanism-based screens has immense potential in the identification of novel anti- nematode drug [95]. FaRPs potently modulate motor function in arthropods, nematodes and platyhelminths and there appears to be at least some homogeneous in the FaRPergic signaling systems. Moreover, there is now increasing evidence of cross-phyla activity for individual FaRPs, providing clear signals of opportunities for target selection and the identification and development of broad-spectrum drugs [96].

ALTERNATIVE DRUGS FROM PLANT EXTRACTS

Extracts of plants, such as papaya and pineapple are known to be effective at killing intestinal nematodes that inhabit anterior sites in the small intestine. It has been demonstrated that cysteine proteinases present in the plant extracts are the active principles [97]. Experiments in vitro using the rodent gastrointestinal nematode H. *polygyrus* have demonstrated that cysteine proteinases of different fruits (papaya, pineapple, fig, and Egyptian milkweed are involved an attack on the structural proteins of the nematode cuticle causing marked damage to the cuticle of *H. polygyrus* adult male and female worms). The efficacy of this mechanism has shown to be comparable for both sexes of worms and was inhibited by the cysteine proteinase inhibitor, E-64 [98]. Further studies confirm that papaya latex has marked efficacy *in vivo* against the *H. polygyrus* this effect shown to be dose-dependent nature (>90% reduction in egg output and 80% reduction in worm burden at the highest active enzyme concentration of 133 nmol) indicating that cysteine proteinases are the active principles *in vivo*. The activity was found to be confined to worms that are in the intestinal lumen. The mechanism of action was distinct from all current synthetic anthelmintics, and was similar as that reported *in vitro* [99], with the enzymes attacking and digesting the protective cuticle. It was demonstrated that treatment had no detectable side-effects on immune cell numbers in the mucosa or on the mucosal architecture. Also similar studies showed that *in vitro* efficacy of these compounds occurs against *Trichuris muris*, and confirm that the cysteine proteinases present in the plant extracts are the active principles [100]. In this work was observed that the mechanism of action of these enzymes involved an attack on the structural proteins of the nematode cuticle, which was similar to that observed with *H. polygyrus*. However, not all plant cysteine proteinases were equally efficacious because actinidain, from the juice of kiwi fruit, had no detrimental effect on either the motility of the worms or the nematode cuticle [99]. Papaya latex was also shown to significantly reduce both worm burden and egg output of mice infected with adult *T. muris*, demonstrating that enzyme activity survived passage to the caecum and was not completely inactivated by the acidity of the host's stomach or destroyed by the gastric or pancreatic proteinases [100]. In humans, the efficacy of an extract of seeds from papaya (*Carica papaya*) has been tested in school children from rural communities in Bolivia [101]. The prevalence of intestinal helminthes was very high among these children being: 44.63% of *A. lumbricoides*; 26.83% of *T. trichiura* and 80.49% of hookworm. The efficacy of the *C. papaya* extract in terms of fecal egg count reduction test (FECRT) two days post- treatment was of 98.62% for *A. lumbricoides*; 99.08% for *T. trichiura* and 89.88% for hookworms. The authors reported that there were no side effects after administration of the treatment. The use of pine apple seeds has been anecdotally reported in many rural communities particularly among Amerindians [102]. Thus the development of novel anthelminthic drugs derived from plants would be very successful in terms of acceptance of rural communities in developing countries. However assessment of the efficacy of plants as anthelminthic may depend on the determination of biologically important levels of reduction of parasitism and it will be required prior to the wide-scale use of plant products for parasite control. Similarly, validation studies in animal and human populations would be required to measure direct anthelmintic effects. In addition some of the active compounds of plants may also have anti-nutritional effects, such as reduced food intake and performance [103], it is essential to validate the anti-parasitic effects of plant products in relation to their potential anti-nutritional and other side effects. A concerted effort on isolation, development, and validation of the effects of these compounds will have to be undertaken before their wider acceptance.

IMMUNOLOGICAL ASPECTS OF *A. LUMBRICOIDES* INFECTION

The immune system of higher organisms has evolved as a complex system of multiple components in order to address the extensive array of invading pathogenic agents. Co-evolution with pathogens is a dynamic and continuing process in which a compromise or tolerance between both host and parasite is generated, enabling the parasite to exist as a low level infection with little harmful effect upon the host. The identification of the many molecules which may be involved in these interactions is essential for understanding processes associated to host susceptibility/resistance against parasite infections.

Immunity against nematode infections has been extensively studied in experimental models (reviewed in [104-106]). Briefly, gut inflammation stimulated by intestinal nematode include cells of both the innate (dendritic cells, mast cells, eosinophils, neutrophils, macrophages and NK-cells) and the adaptive immune system (CD4 T-lymphocytes and often antigen-specific IgE secreting B-lymphocytes. The innate and adaptive cells are of Th2 class which secret preferentially IL-13 and IL-4 cytokines. Pro- inflammatory cells like mastocytes and eosinophils stimulated by these cytokines may release many mediators (heparin, reactive lipids or eicosanoids, and enzymes including tryptase and chitinase) leading to increased permeability of blood vessels, increased mucus production and smooth muscle contraction. These mechanisms may contribute to make a hostile microenvironment to the parasite promoting worm expulsion. It has been proposed that intestinal helminthes skew the immune system to a Th2-type by the stimulation of innate immune mechanisms through the release of particular molecules**.** For example proteases prominently secreted by different life stages of helminths cause epithelial damage and thus release of Th2 stimulating thymic stromal lymphopoietin (TSLP) leading to loosening of epithelial tight junctions and direct cytokine and chemokine release from mast cells and eosinophils that strongly prime Th2 responses [107]. Also proteases can act directly through protease activated receptors (PARs) that lead to the non-specific amplification of Th2 actions type response [108]. Both innate and adaptive Th2 mechanisms are mutually supportive. It is accepted that the maintenance of Th2-type inflammatory reactions is by Th2 cytokines where IL-13 may play in particular a central role, acting through the STAT6 molecule [21]. This may be supported by data indicating that genetic knockout of STAT6 renders mice incapable of clearing helminth gut infections [109]. In humans, infection by *A. lumbricoides* has been associated with a polarized Th2-type immune response characterized by elevated levels of serum immunoglobulins particularly IgG4 and IgE (reviewed in [110]). The role of humoral immune responses in infection has been controversial. Early studies carried out in different populations from endemic areas have suggested that antibody responses correlate with the intensity of the infection [111] and thus may not confer protection against reinfection [112] whereas immunoepidemiological studies carried out in Venezuelan urban slum children indicated that protection from re-infection was associated with high levels of *A. lumbricoides* specific IgE [113]. Further studies assessed in Venezuelan rural children had shown that atopic children which have a great capacity to produce IgE are more protected against re infection after anthelminthic treatment [13,114]. Other approaches testing human Th2-type cytokine responsiveness in relation to future susceptibility to *A. lumbricoides* and *T. trichiura* infection were able to show that persistently susceptible individuals were characterized by a weak Th2-type cytokine response, thus indicating a protective function of such responses [115]. The immune response against *A. lumbricoides* is very complex and may involve a number of host intrinsic variables which in turn may be influenced by social, cultural and environmental factors. This situation probably reflected the differences observed in results obtained from studies carried out in very distinct populations. In addition, as parasites are under constant attack by a range of effective immune mechanisms, they have developed effective evasion mechanisms that also may influence the capacity of the host to mount effective responses against infection. The process of parasite evasion may vary from simple avoidance to a more active modulation of the immune response in order to establish a non-inflammatory environment that allows the parasite to survive. Nematode parasites may enhance survival by directing the immune response to that of a less appropriate type. For example interference with the Th1/-Th2 response balance, general suppression of T and B cell responses, mimicry of host proteins which direct the immune response and even the use of cytokines as parasite growth factors have been reported (reviewed in [116,117]). In humans, *A. lumbricoides* infection has been associated with immune hyporesponsiveness and T-cell anergy [115,118]. Apart from the suppression of immune responses, worms may also subvert host immunity through the induction of inappropriate effectors. It has been suggested that the induction of high levels of polyclonal IgE by *A. lumbricoides* may modulate immediate hypersensitivity reactions by competitive binding of FcεR1 receptors interfering with the process of worm expulsion [119]. Competitive blocking of epitopes by IgG4 may also be important in compromising specific IgE responses against the parasite [120]. The majority of these studies have been performed using somatic extracts; pseudocelomic fluid of *worms* as well as excretory secretory products from larvae stages and little is known about the particular immunoreactive antigens involved in the stimulation and maintenance of such responses. Much of the effort at elucidating the nature of these molecules have been done in experimental models in which specific antigens associated to a variety of vital parasite functions have been identified and some of them are currently used for vaccine development purposes.

LIPID-BINDING PROTEINS

It is well known that *A. lumbricoides* contains appreciable quantities of lipids. In females at least part of the lipids may be destined for incorporation into eggs that need lipids for their energy metabolism and for the synthesis of membrane lipids and lipid mediators. Fatty acids are involved in various cellular processes by their interaction with enzymes; membranes; ion channels; receptors and genes. Their utilization by cellular organelles requires facilitation of fatty acid solubility and transport in which fatty acid binding proteins must be involved being those proteins of great importance for *worm* survival [121]. One of the most studied is the *A. lumbricoides* body fluid antigen-1 (ABA-1). This molecule has been characterized as the most abundant protein in the pseudocelomic fluid of the parasite. It is also release by tissue invasive larvae. In addition, mRNA for ABA-1 of *A. lumbricoides* is present in infective larvae within the egg and in all parasite stages but it is not detectable in un- embryonated eggs. ABA-1 mRNA is confined to the gut of adult worms and not in body tissues [122] and it has been found that binds retinol A as well as other fatty acids [123]. ABA-1 protein constitutes the most important of the group of antigens of the denominated family of nematode polyprotein allergens (NPA) for its capacity to stimulate strong IgE responses [124]. There is strong evidence that ABA-1 plays an important role in the control of human ascariasis. It has been

reported that elevated IgE levels against ABA-1 protein are associated to natural immunity against the parasite in groups of African children [125]. Also studies performed in an hyper endemic Cameroonian rural community have shown that IgE against rABA 1 is associated to acquired immunity in older children (>12 years) and adults [120]. Recognition of ABA-1 may have a genetic basis. Experimental models have indicated that immune responses against ABA-1 are HLA restricted in mice [16] although HLA associations with antibody responses against this protein have not been reported in humans. Recent work performed in Colombian endemic areas [126] have shown that polymorphisms of LIG4 and TNFSF13B of the 13q33 region are associated with high levels of specific IgE to ABA-1 in *A. lumbricoides* infected children confirming earlier studies implicating the TNFS13B gene on chromosome 13 in resistance /suscep-tibility to this infection [18]. In addition more recent work have indicated that rABA-1 has no cross reactivity with other environmental allergens such as house dust mites and it has been proposed that ABA-1 allergen may be and specific marker of *A. lumbricoides* infection [127]. Taking together these results may suggest the usefulness of ABA-1 to identify susceptible/resistant individuals among infected people in endemic areas.

TROPOMYOSIN

Tropomyosin is a microfilament associated protein present in all eukaryotic cells. It is found in multiple isoforms that are characteristic for specific types of tissue. It is essential in the process of muscle work, proper action of the movement apparatus and the basic functionality of filaments within the cytoskeleton. There is a high degree of homology among tropomyosins, even of phylogenetically distant species of invertebrates, but not with vertebrate tropomyosins. Invertebrate tropomyosins induce IgE antibodies and are potent allergens for humans whereas vertebrate ones were reported to be non-allergenic [128]. Tropomyosin from *A lumbricoides* presents a high degree of sequence identity to those from other invertebrates, including cockroach, mites, and shrimp [129]. It is expressed in high levels in the third stage larvae (L3), which is the stage of pulmonary passage of the parasite and it has been reported that stimulates strongly the production of IgE [129]. It has been proposed that IgE responses to tropomyosin derived from inhalant allergens such as cockroaches cross-react with *A. lumbricoides* tropomyosin in atopic individuals living in endemic areas [130] therefore promoting the development of respiratory symptoms and asthma observed in these populations [4,130]. On the other hand, enhanced IgE-mediated inflammation seen in atopic individuals correlate with protective immunity to ascariasis [125] suggesting a role of these allergenic proteins in the stimulation of protective pro-inflammatory mechanisms. However, evidence from experimental models has indicated that the protection conferred by tropomyosin in animal vaccine trials may be not necessary associated to its allergenic properties [131]. Comparative studies performed in the jird model showed that the protective effect of tropomyosin against challenge with *A. viteae* L3 depends on the conditions of immunization [132]. Thus immunization with a recombinant *A. viteae* tropomyosin together with the STP adjuvant (an adjuvant based on a mixture of squalane, Tween and pluronic) or vaccination with its cDNA- both inducing predominantly Th1 responses, resulted in protection of jirds in terms of reduction of worm burdens. By contrast, immunization with recombinant or native tropomyosin together with alum favoring Th2 development did not protect against challenge infection. However, further studies are needed to elucidate the impact of adjuvants on the type of immunity elicited by allergenic proteins and its importance in the stimulation of protective immune responses against nematode parasites as well as in the development of future vaccines.

NEMATODE PROTEASE

Nematode proteases constitute a group of highly evolutionary conserved molecules among distinct species of nematodes. They are involved in different vital functions such as digestion of structural proteins facilitating extensive migration through host tissues; parasite feeding; molting and larvae development [133]. There is evidence that protease activity is associated to the stimulation of innate immune mechanisms leading to the development of highly pro-inflammatory Th2-type immune mechanisms. Basophils which are able to secrete IL-4 *in vitro* following stimulation with allergens and helminth products are thought to be the bridge between innate recognition of nematode proteases and the initiation of adaptive Th2 cytokine responses [134]. This hypothesis can be supported by experimental work showing that mice immunization with papain results in the transient recruitment of basophils to lymph nodes that peaked 1 day prior to the peak of IL-4 producing CD4+ T cells. The papain-elicited basophils within the lymph node were shown to express thymic stromal lymphopoietin (TSLP) [135]. This cytokine which is produced predominantly by epithelial cells has been implicated in the differentiation of Th2-type immunity [107]. *In vivo* depletion of TSLP or basophils correlated with impaired Th2 cell differentiation following papain immunization, suggesting a role for basophil-derived TSLP in papain mediated Th2 cytokine responses [135]. In addition there is evidence that protease receptors PARS constitute a recognition receptor for nematode-derived proteases and that these molecules may play an important role in triggering innate protective immune mechanisms in the host [136]. Moreover, studies carried out in experimental models using the infection *N. brasiliensis* in mice have confirmed the role of protease-activated basophils as an important innate immune mechanism leading to worm expulsion [137].

Regardless their role in innate immunity nematode protease may function as potent antigens and can be associated to protection against nematode infection. For example, early experimental models have indicated that cysteine proteases derived from somatic extracts and excretory secretory products of the gastrointestinal nematode *Nypostrongylus brasiliensis* stimulate the production of IgE [138] that promotes early worm expulsion in mice [139]. Studies using proteomic analysis of the excretory secretory products (ES) from adult worms and the fourth larval stage (L4) of *Teladorsagia circumcincta*, a parasite of sheep, revealed the presence of several metabolic enzymes that were present in both adult and larvae excretory secretory products [140]. L4 ES alone contained several proteins like ioredoxin peroxidase, an enzyme that can detoxify free radicals resulting from host inflamma-

tory responses to the parasite, a cysteine proteinase which may enable penetration of the gastric mucosa and 2 different galactins associated to cell differentiation and morphogenesis. Adult ES contained a nucleoside diphosphate kinase homologue, an enzyme which has been linked to cellular changes and can affect liquid secretion and goblet cell degranulation [140] that may contribute to worm expulsion. Proteomic analysis of human *A. lumbricoides* ES products have not been carried out. Nevertheless we may suggest that would be a suitable approach to identify a great source of protective enzymes with the capacity to interact with the host immune system. Also the use of genomic approaches may provide with a lot of new information about the role of these enzymes. A recent study has analyzed various transcribed genomes from *A. suum*, *H. contortus* and *C. elegans*. A large collection of intestinal sequences from these parasites was generated and based on sequences similarities a group of 241 well conserved intestinal proteins (IntFam241) was identified [141]. These proteins accounted for 20% of the sampled intestinal transcriptomes from the three nematodes and are proposed to represent conserved core functions in the nematode intestine. Functional characterization of the IntFam-241 suggested important roles such as protein kinase and protease as well as important metabolism pathways [141] that may provide of numerous targets for further studies of immune interactions. For example, previous studies have shown that the aspartic protease hemoglobinase APR-1 and the cysteine protease-hemoglobinase CP-2 from hookworm, involved in degradation of hemoglobin in the worm digestive tract [142] are promising protective antigens against parasite infection in laboratory dogs in terms of reduced host blood loss and fecal egg count [143].

On the other hand, Islam *et al.* [144] proposed that pyrophosphatase, which is an important protease involved in molting processes, may play an important role in protection against *A. suum* larvae migration. Experiments using reverse transcriptase PCR analysis, have demonstrated that disruption of AsPPase gene function by RNA interference resulted in suppression of AsPPase mRNA levels, native protein expression and inhibition of molting of third-stage larvae (31%). In the same study, Immunization of mice with Anti-recombinant pyrophosphatase (rAsPPase) immunoglobulin G (IgG) also resulted in 57% inhibition of molting of A. suum lung-stage third-stage larvae to fourth-stage larvae *in vitro* causing developmental arrest. Mice immunized with rAsPPase exhibited high antigen-specific IgG antibody responses and were protected (>70%) against a challenge *A. suum* migratory-phase infection.

HUMAN VACCINES

The development of a vaccine against *A. lumbricoides* has not been achieved, however and probably due to the greater impact on children's health, a vaccine against human hookworm is currently being developing. Nematode proteins involved in tissue invasion are particularly good candidate antigens for the development of vaccines. Therefore, and on the basis of *in vitro* data, animal trials and human epidemiological studies the protein known as (*Na*-ASP-2), secreted by hookworm larvae (L3) was selected as a vaccine antigen to undergo further development [145]. Studies carried out in dogs vaccinated with *A. caninum* irL3 demonstrated that ASP-2 is the predominant antigen to which the protective antibody response is directed [146]. Vaccination with recombinant *A. caninum* ASP-2 results in the stimulation of high levels of antigen-specific IgG as *well as* a high degree of protection after challenge with live L3, evidenced by reduction in adult worm burdens, fecal egg counts, and host blood loss, when compared with control animals [146]. It was shown that Anti-ASP-2 IgG from vaccinated animals also inhibited the *in vitro* migration of larvae through tissue [147]. Finally, studies of populations living in areas of Brazil and China where hookworm infection is endemic demonstrated that anti-ASP-2 antibodies are associated with a reduced risk of acquiring severe where hookworm infection [145]. Currently ASP-2 is on phase 2 trial in healthy adult volunteers from Brazil [22,143]. This is the first attempt to develop a vaccine against human intestinal worms and means a hope for similar approaches for the control of *A. lumbricoides* and other intestinal parasites that represent the most common infections of the world's poorest people.

On the other hand, studies including molting processes and larvae development in *A. suum* experimental models, using permissive hosts and also natural infection in pigs have led to the identification of different proteins that can be potent candidate vaccines for *A. lumbricoides* infection. This presumption is based on evidence obtained from several work that have characterized the two species of parasites on morphological, immunological, and biochemical bases demonstrating its great homology [148] supporting the extended use of *A. suum* antigens in the study of *A. lumbricoides* immunity. In addition, *A. suum* of swine origin can develop in human hosts, indicating its possible emergence as an important zoonotic parasite [149]. Therefore, studies carried out by Tsuji *et al.* [23] have identified a potent protective 16-kDa antigen (As16) that is shared by both the human and pig roundworms but has no similarity to mammalian proteins that is currently being using for the development of experimental mucosal vaccines against *A. suum* [23]. Mucosal vaccines are considered the most suitable type of vaccines to combat emerging and re-emerging infectious diseases because of their ability to induce both mucosal and systemic immunity. Considerable advances have been made toward the development of mucosal vaccines against influenza virus and rotavirus. Many additional mucosal vaccines are in development, including vaccines against cholera, typhoid, traveler's diarrhea and respiratory infections. In addition to oral and nasal vaccines, transcutaneous (or skin patch) and sublingual immunizations are now part of a new generation of mucosal vaccines (reviewed in [150]). Taking in account the advantages of these routs of immunizations, the effect of intranasal immunization of pigs with purified rAs16r on molting processes and larval survival have been studied [23]. It was found that immunization with rAs16 induced a type II immune response characterized by elevated levels of interleukin (IL)-4 and IL-10 and High titers of rAs16-specific mucosal IgA and serum IgG antibody. It was observed that immunized pigs were protected against the larval migration of the *A. suum* tissue phase. Also pIg-rAs16 was capable of interfering with larval survival thus inhibiting invasion of the parasite into the organs and tissues of pigs. The authors proposed that rAs16 will contribute to the ongoing development

of mucosal vaccines against *Ascaris* species for both human and veterinary uses.

In addition in recent years, successful expression of antigens in plants and oral immunization has been reported for antigens obtained from various infectious pathogens (reviewed in [151]). Vaccine production in rice seeds in particular has many advantages such as high expression levels, large biomass and low production costs [152]. Rice is cultivated in large farms especially in developing countries. Glutelin is a major seed storage protein of rice accounting for about 80% of the total seed protein [153]. Also many recombinant foreign proteins such as soybean glycinin, ferritin or the Japanese cedar pollen allergens have been highly and efficiently produced in rice grains using glutelin promoter [154,155]. Preliminary studies to produce a candidate protein for rice plant-based edible vaccines against *A. suum* are being carried out by Nosoye *et al.* [156]. For these purposes they are using a 14-kDa protective surface antigen of *Ascaris suum* L3 larvae and its fusion chimera with a mucosal carrier molecule cholera toxin B subunit (CTB) which is produced in rice (*Oryza sativa* L) under the control of the endosperm-specific glutelin-B promoter. This model may lead to the generation of transgenic seeds containing a vaccine candidate antigen. Further studies are needed to test the viability of this system and its useful in the development of more suitable vaccines for animal and human ascariasis.

PARASITE-DERIVED MOLECULES INVOLVED IN IMMUNE REGULATION

Several studies carried out in distinct experimental models have revealed a number of active molecules in extracts of helminths and in ES products that can modulate the immune system of the host [157]. The main function of these molecules has been associated to parasite immune evasion and some of them may be considered as target for drugs and vaccine. However, because of their suppressor properties they have also been considered as a great source of new drugs to treat other diseases [158].

PAS-1 OF *A. SUUM*

Early work conducted by Itami *et al.* [159] have demonstrated that high molecular weight components purified by gel filtration chromatography from an *A. suum* adult worm extract were able to suppress the murine antibody production to a bystander antigen. This effect was attributed to a 200 KD a protein component called PAS-1. The protein was affinity purified using a monoclonal antibody (MAIP-1) produced against high molecular weight suppressive components. Pas-1 was shown to be capable of down regulating antibody production Th2 secretion; eosinophils recruitment and airway hyper-responsiveness induced by *A. suum* allergens [160]. This effect was mediated by the stimulating capacity of Pas-1 on the production of regulatory cytokines such as IL-10 [160]. Therefore Pas-1 has shown to be very efficient in blocking allergen responses in mice lungs and in this sense this protein would be of great pharmacological importance. On the other hand, due to the strong anti-allergenic properties of Pas-1 future studies directed to its role in controlling pro inflammatory pathways in human *Ascariasis* may be highly relevant.

PROTEASE INHIBITORS

As stated above proteases are central to normal physiological processes within the parasite. Nevertheless its activity must be tightly regulated to avoid serious damage on the parasite and host. Parasite proteases are likely to have arisen from a relatively limited set of peptidases with broad substrate specificities in the early days of life and evolved by a process of gene duplication and divergence to acquire a high degree of substrate specificity thus preventing collateral damage [157]. Also regulation is achieved using a diverse array of protease inhibitors of both host and parasite origin. Thereby inhibitor's principal function may be simply to interact with parasite proteases to prevent aberrant activity ant to minimize damage in the micro environment in which protease activity develops. However, protease inhibitors can also modulate the host immune response to the parasite inhibiting a large number of pro inflammatory pathways.

Probably the best-characterized groups of helminth immunomodulators are the cystatins (cysteine protease inhibitors). Most of the studies underling their importance on immune regulation has been carried out in filarial nematodes (reviewed in [161]). It has been demonstrated that nematode cystatins inhibit, among others, proteases involved in antigen processing and presentation, interfering with antigen-specific T cell responses and the proliferation of T and B cells [162]. They can also modulate cytokine responses. Particularly they are involved in the up regulation of IL-10 that leads to the down regulation of co-stimulatory surface molecules of macrophages [163]. These properties contribute to induction of an anti-inflammatory environment, concomitant with a strong inhibition of cellular proliferation. The possible role of Cystatins in *A. lumbricoides* infection has not been identified, nevertheless nippocystatin a 14-kDa cystatin family 2 protein from the intestinal nematode *Nippostrongylus brasiliensis* has shown to selectively inhibited cysteine proteases and also was found to be capable of modifying antigen processing thereby modulating antigen-specific immune responses [164] suggesting that intestinal nematodes also utilize this protease inhibitor to evade the host defense system contributing to the persistence of the infection.

The serine protease inhibitors are of great pharmacological importance because of their role in regulating the endogenous proteinases of blood clotting, clot resolution and in inflammation [165]. A detailed description of their sequence, structural motifs and mechanism of binding has been reviewed by Rawlings *et al.* [166]. The main function of these inhibitors is the prevention of proteolysis by host proteases thereby contributing to the survival of the parasite. For example protease inhibitors from aqueous *A. lumbricoides* extracts can inhibit trypsin [167], chymotrypsin [168], elastase [169], carboxypeptidase A and B [170] and the pancreatic enzymes pepsin and gastricsin [171]. Also As*caris* aspartyl protease (PI-3) inhibits pepsin protecting the worm from proteases digestion in the host gastrointestinal tract [172] and there is evidence that PI-3 also inhibits cathepsin E and antigen processing by T-cells, suggesting its immunomodulatory function [173]. Other studies have identified and characterized a serine protease inhibitor (Ov-SPI-1) from O. *volvulus*, homologous to the *A. suum* chymotrypsin and trypsin inhibitors [174]. Localization studies indicated an association with

molting, confirmed by RNA interference studies. In addition, the protein was localized to the sperm and eggshells, indicating a reproductive function. Finally, the functionally active recombinant protein inhibited neutrophil cathepsin G amongst others [174] thus suppressing an important mechanism for killing the larvae that may be shared by various nematode species. Another study has shown that a secretory chymotrypsin/elastase inhibitor from *Trichuris suis* can inhibit the capacity of mast cell chymase to activate IL-1, increase epithelial cell permeability and stimulate inflammatory cell recruitment [175] suggesting that serine protease inhibitors may exert anti-inflammatory functions through the down regulation of mast cell function. On the other hand pharmacological studies have demonstrated the potent anti-inflammatory properties of serine protease inhibitors. For example the serine protease, C1 esterase inhibitor has been used effectively in a wide range of inflammatory conditions including bacterial sepsis, cytokine-induced vascular leak, pancreatitis, and transplant rejection [176,177]. *In vitro* studies demonstrate that C1 esterase inhibitor can block T-lymphocyte activation, proliferation and generation of cytotoxic T lymphocytes [178]. The serine protease inhibitor aprotinin has been found to have some efficacy in the treatment of trypsin induced shock [179]. Serine protease inhibitors have also been used to treat atopic dermatitis. Synthetic and commercial specific chymase inhibitors: SUN-C8257 and SUN-8077 have been shown to reduce dermatitis in animal models [180,181]. SUN-C8257 was shown to reduce accumulation of inflammatory cells including mast cells and eosinophils in the skin [181].

It has been demonstrated that metalloprotease inhibitors in humans have an important role in regulating matrix metalloproteinases [182]. The first tissue inhibitor of metalloproteinase (TIMP) homologue of parasite origin was cloned from an adult *A. caninum* cDNA library by immunoscreening with anti-hookworm ES antiserum [183]. It has been proposed that the activity of matrix metalloproteinases inhibitors is associated to intestinal injury repair caused by hookworms when they attach to the intestinal mucosa [183]. This type of action could modulate ulcer development at the site of attachment limiting worm damage to the host. Also there is evidence that hookworm metalloprotease inhibitors may inhibit neutrophil collagenase activity and thus regulate host inflammation at the attachment site [184]. Other studies using intensity-fading MALDI-TOF mass spectrometry have identified a metallocarboxypeptidase (MCP) inhibitor in protein extracts from *Ascaris* nematodes. It was denominated as *Ascaris* carboxypeptidase inhibitor (ACI) [185]. Its function has been associated with the inhibition of the activity of mast cell derived metalloproteases in the intestinal mucosa of the host. These data are consistent with the specific localization of ACI in the intestine and body wall of male and female *Ascaris* worms and in fertilized eggs. Such localization is compatible with the protection of adult worms and eggs in the host intestine and of the larvae during migration. Therefore, this protease inhibitor may play an important role in allowing *Ascaris* to survive in a hostile environment by evading or dampening pro- inflammatory host responses that might otherwise kill it or trigger its expulsion.

Nematode serine protease inhibitors have shown to be highly antigenic and they have been implicated in the stimulation of protective Th-2 responses such as the production of IgE and IgG4 [186,187]. It has been proposed that serine protease inhibitors are associated with the non-specific polyclonal stimulation of IgE [188]. The mechanism by which these protease inhibitors may stimulate such responses is not well understood. It has been reported that the protease inhibitor α1-antitrypsin that is abundant in *A. lumbricoides* extracts [173] induce a potent and selective dose dependent increase of IgE and IgG4 production by human tonsillar B cells stimulated with IL-4 and anti-CD40 antibodies *in vitro* [189]. This effect was also accompanied by an increase in germ line and mature e mRNA transcription and the expression of membrane CD23 (the low affinity receptor for IgE) and its ligand CD21. The authors proposed that alpha 1 trypsin inhibitor prevents the cleavage of CD23 favoring cell-cell interactions between CD23+ and CD21+ B cells thus inducing the non-specific stimulation of IgE. This is consistent with studies carried out in children from endemic rural areas in which high levels of total IgE correlates with the number of circulating CD20+ CD23+ B and CD20+CD21+ B cell sub populations and also with the intensity of the infection [114] suggesting that, nematode serine protease inhibitors would be involved in the stimulation of inappropriate immune responses, contributing with the persistence of the infection.

GLYCOPROTEINS AND GLYCOLIPIDS

The carbohydrates linked to proteins and lipids of nematodes have attracted significant attention in the past years due to their immunogenic and immunomodulatory properties particularly those of phosphorylcholine (PC)-modified carbohydrates [190].

It has been reported that the relevant nematode PC-substituted oligosaccharides occur in two different groups: the first group occurs as PC-modified glycosphingolipids such as those found in *A. suum* [191], *A. lumbricoides* [192], *O. volvulus* [193], and in *C. elegans* [193]. In these organisms the glycolipid-bound PC is linked to an N-acetylglucosamine residue additionally, in the case of *A. suum* glycolipids, phosphoethanolamine has been also detected [191]. In the second group, PC-containing protein-linked N-glycans have been found in *C. elegans* [194,195], *Ac. viteae* [196], *T. spiralis* [197] and *O. volvulus* [198]. These N-glycans contain the typical trimannosyl core, with and without core fucosylation, and carry between one and four additional N-acetylglucosamine residues. In these PC-modified glycans, the core fucose is a1,6-linked as in mammals. Other N-glycans from nematodes also carry a1, 3-fucose on the proximal, and uniquely, distal GlcNAc residues of the core [198]. Structural features of glycolipids including oligosaccharide backbone, substitution with PC, and ceramide composition are shared between all the parasitic nematode species [190,199] with widespread anatomical location in the worm, suggesting the importance of these components in host parasite interactions.

There is evidence that N-glycans of distinct species of nematode as well as other helminths have the capacity to induce Th2 type immunity [200]. Moreover patients with pollen, food and venom allergies exhibited IgE specific to N-glycan epitopes carrying core xylose and/or core α1,3-fucose

[201]. Immunological cross reactivity between different carbohydrate epitopes of different species of plants and invertebrates have been also reported [201] suggesting that these IgE stimulating glycans may represent a fundamental class of ligands widely distributed in nature.

The immunomodulatory activity of nematode glycolipids has been shown to be associated with phosphorylcholine (PC). The secreted filarial nematode glycoprotein ES-62 constitutes a suitable example. Through PC modifications, ES-62 can inhibit the proliferation of CD4+ T cells and conventional B2 cells *in vivo*, and reduces CD4+ cell IL-4 and IFN-γ production [202]. Conversely, ES-62 promotes proliferation and IL-10 production by peritoneal B1 cells [203]. It has been proposed that inhibition of proinflammatory Th1 responses occurs as ES-62 interacts with toll-like receptor (TLR) 4 through its PC residues [204], also in mast cells the interaction of TLR 4 with ES-62 results in the inhibition of degranulation and release of inflammatory mediators [205].

In contrast PC substituted glycosphingolipid of *A. suum* were shown to stimulate rather than suppress the human peripheral blood mononuclear cells (PBMC) production of TNF alpha and IL-6 cytokines in a dose response range similar to that of lipopolysaccharide's (LPS). Removal of the PC substituent by treatment with hydrofluoric acid completely abolished this biological activity [206]. The mechanisms by which these glycosphingolipids induce cytokine production are not clear. The authors proposed that they may act in a direct way by replacing intracellular lipid second messengers such as ceramide.

In addition to proteins, glycolipids can be target of antibody responses. In the case of helminths antibody reactivity to lipids has been described in schistosomiasis [207] and more recently in *A. lumbricoides* infection [192]. Epidemiological studies using *Ascaris* derived glycolipids have shown that children carrying heavy infections show highest IgG reactivity glycolipids compared to lightly or non-infected children [192]. In the same study IgG antibody reactivity to both glycoproteins and glycolipids were directed to the PC moiety as determined by either removal of this group or a competition assay. This was most pronounced for glycolipids, where removal of the PC moieties by hydrofluoric acid treatment abrogated IgG antibody reactivity. Measurement of IgG4 and IgE isotypes showed no IgG4 reactivity to *Ascaris* specific glycolipids whistle elevated IgE responses were detected in subjects with light or no *A. lumbricoides* infection. These results indicate that *A. lumbricoides* specific glycolipids have antigenic properties and are involved in the control of parasite burden. The mechanism by which glycolipids can stimulate IgE and IgG responses is not clear. The authors proposed that antibodies could develop directly to glycolipids through activation of CD1d which is a non-classical MHC lipid presenting molecule. Nevertheless, cross reactivity between glycolipids and PC present on proteins may also occur [192]. The immunomodulatory effects exhibited by PC- substituted molecules can be seen as a contribution to equilibrium in host -parasite interactions in which expanding of Th2-type responses enables the parasite to survive preventing harmful pro-inflammatory mechanisms in the host. Since PC substituted molecules from nematode differ clearly from those from the host, they would be a suitable target for the development of new anthelminthic strategies.

CHITIN

Chitin is one of the most abundant natural polymers on earth and is found in fungi, protozoa, insects, crustaceans and parasitic nematodes. It is the second most abundant biopolymer in nature, with estimates of billions of metric tons produced annually in oceans alone. Chitin provides osmotic stability and tensile strength to fungal cell walls and scaffolds the rigid exoskeleton in insects [208]. Nematode chitins are important for egg shell integrity and for structure of the rigid pharynx, including the buccal cavity and grinder, a specialized cuticle that is shed and re-synthesized during molting [209]. The mammalian counterpart of chitin has not been described. As some chitin derivatives are known to be nontoxic, non-allergenic, biodegradable, and biocompatible, a number of prostheses such as artificial skin, contact lenses, surgical stitches have been produced from chitin derivatives and are widely used in medical practice [210].

Experimental models using the infection with the migrating helminth, *Nippostrongylus brasiliensis* to examine lung tissue responses to chitin have shown that the intranasal administration of chitin induced an accumulation of eosinophils and basophils [211]. Exposure to chitin also stimulated the alternative activation of macrophages as indicated by the presence of arginase-expressing cells in the lung as early as 6 h post intranasal administration of chitin [211]. The recruitment of innate immune cells has shown to be dependent both upon expression of the high affinity receptor for leukotriene B4, BLT1 and upon the presence of macrophages [211], suggesting strongly a possible role of chitin in innate immune cell recruitment, leading to preferential Th2-type responses and raises the possibility that chitin can be directly involved in the generation of allergic responses. In addition highly Stat6-dependent genes included acidic mammalian chitinase (AMCase) and Ym1 and/or Ym2 are induced in the lungs of mice during *N. brasiliensis* infection [211] this is consistent with previews studies demonstrating that a number of chitinase or chitinase-like proteins are ubiquitously expressed in the airways and intestinal tracts from insects to mammals (reviewed in [212]). In addition, regardless their role in allergic diseases other studies have shown that chitinase family proteins confer protective functions to the host against exogenous chitin-containing pathogens and may also be associated to tissue remodeling [213]. Further studies are need for elucidating whether these molecules have a role in gastrointestinal parasite infections. If this is the case and due to their immunogenic properties these molecules may be an important target for therapeutic approaches.

CONCLUDING REMARKS

Gastrointestinal helminth infections, *A. lumbricoides* being the most prevalent, are among the most important neglected tropical diseases affecting particularly children populations. Even though the efficacy of current drugs has been proven, high prevalence of this infection as well as elevated rates of re-infection following single-dose treatment have persisted, indicating that repeated doses of massive treatment will be required for eradication programs based solely on

pharmacological approaches. This situation may lead to drug resistance, highlighting the importance of new drug development. However, only a few new drugs seem to be available in the upcoming years. The increased availability of helminth parasite genomic research directed at the identification of parasite-derived molecules involved in vital functions may enhance the capacity to develop new drugs. On the other hand, the most sought-for prophylactic strategy is vaccination due to the increasing incidence of anthelmintic resistance with slow progress towards the discovery of novel drugs. Nevertheless, the development of efficient antiparasitic vaccines was shown to be a far greater challenge than in the case of bacteria or viruses. This is partly a result of the complex immunological interactions occurring during intestinal helminth infections, which are not yet fully understood, especially regarding the immune mechanisms conferring protection. Most of the studies have been carried out in experimental models and very little is known about immune mechanisms in human hosts. Therefore, more extensive studies are need for the identification of suitable antigens for vaccine targets. Alternatively, there is a lot of interest in the identification of nematode-derived immune regulatory molecules, an indispensable condition to understand parasite survival strategies that would be important for the design of new anthelmintic approaches and, additionally, may offer a source of new mediators for the control of inflammatory pathways of many other diseases. Another problem is progressing from the research phase of vaccine development to commercial production and marketing that would be an important economic challenge for developing countries in the upcoming years.

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