

Review Article

Expression of helminth genes in *Leishmania*: an experimental transfection system to test immunological function

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SUMMARY

Functional analysis of genes from parasitic helminths requires, at the present time, heterologous expression. We have adapted the well-characterized system of transfection in *Leishmania* protozoal parasites, as a means of analysing the effect of single filarial genes on the mammalian immune system. For example, testing the function of the *Brugia malayi* abundant larval transcript (*ALT*) gene-transfected *Leishmania mexicana* were found to be significantly more virulent in macrophages in vitro. The course of infection in vivo is also aggravated by expression of the *ALT* gene. Examples are also given of transgenes which reduced in vitro growth within macrophages, as well as others which exert no effect on the protozoal parasitism. Thus, *Leishmania* transfection provides a tractable system to analyse helminth gene function within the context of the host immune system.

Keywords filarial nematode, immune modulation, macrophage, transgenesis

Successful parasites are adept at sophisticated manipulation of the host immune system, thereby avoiding elimination by immune effector mechanisms. In many microbiological systems, the exquisite interplay between host and pathogen has highlighted precise mechanisms of interference with MHC-dependent antigen presentation (1) and pathways of intracellular immune signalling (2). For helminths, similar analyses of immune modulation at the molecular level are relatively sparse. To some extent, this reflects a more complex life history, with successive stages occupying differing host niches, and a more complex antigenic profile within metazoan pathogen organisms (3). However, it is the difficulty of genetic manipulation of helminths which has presented the major obstacle to dissecting gene function (4).

The helminth group of parasites is polyphyletic and displays an extreme diversity of developmental and biological characteristics which impede the transfer of successful techniques from the free-living *Caenorhabditis elegans* to the many pathogenic species. Methods for transfection of exogenous genes are now being developed for Schistosomes (5–7), but among the parasitic nematodes, heritable transfection technology has been developed only for two species which develop through a free-living cycle, *Parastrostrongylodes trichosuri* (8) and *Strongyloides stercoralis* (9,10). For most pathogenic species, in which adult nematodes are lodged within the vertebrate host, only transient transfection by ballistic methods have been reported (11,12). Gene knockdown by RNA interference (RNAi), however, has made more rapid progress: since the first report of successful RNAi in *Nippostrongylus brasiliensis* (13) as well as in plant parasitic species (14), the technique has been applied to *Ascaris suum* (15), *Brugia malayi* (16), *Haemonchus contortus* (17), *Onchocerca volvulus* (18,19), *Trichostrongylus* (20), *Litomosoides sigmodontis* (21) and *Schistosoma mansoni* (22,23). The increasing refinement and success of this approach, and its applicability to a range of target species, augurs well for general use; however, understanding the role of a target

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Received: 31 July 2007

Accepted for publication: 11 September 2007

Table 1 Summary of immunological phenotype of transfectant *Leishmania* expressing helminth genes.

Helminth species	Helminth gene	Macrophage infection <i>in vitro</i>	Murine infection <i>in vivo</i>	Reference
<i>Brugia malayi</i>	<i>Bm</i> -ALT-1	2–3× higher	More virulent	(52)
	<i>Bm</i> -ALT-2	2–3× higher	More virulent	
	<i>Bm</i> -ALT-2-ADD	As wild-type	As wild-type	
	<i>Bm</i> -CPI-2	As wild-type	As wild-type	
	<i>Bm</i> -MIF-1	2–3× lower	As wild-type	L. Prieto-Lafuente, unpublished observation
	<i>Bm</i> -MIF-2	2–3× lower	As wild-type	
	<i>Bm</i> -VAL-1	As wild-type	nd	J. Murray <i>et al.</i> , unpublished observation

nd = not determined.

gene also requires some means of transgenic expression for a spectrum of functional studies to determine downstream targets, binding partner proteins, quantitative (gene dosage) effects under inducible promoters and site-directed mutagenesis analysis of potentially key residues and domains.

In the absence, at the present time, of robust genetic methods for functional genomics within parasitic helminths, heterologous transfection approaches offers the only way forward. Parasite nematode genes can successfully be expressed in the free-living and genetically tractable nematode *C. elegans*. These have demonstrated conservation of function, for example, that a *H. contortus* gene can rescue a *C. elegans* mutant in a homologous locus (24), and that a *St. stercoralis* transcription factor can restore insulin pathway induction of dauer formation in *daf* mutants (25). A similar approach permitted functional studies, for example, of temperature sensitivity of a parasite heat shock protein expressed in *C. elegans* (26). In addition, parasite promoter elements from *H. contortus* (27) and *B. malayi* (28) are successfully recognized in *C. elegans* allowing definition of promoter elements and tissue specificity. This illustrates the power and precision of transfection in *C. elegans* – but one must also recognize the limitations, in that a property not present in *C. elegans* (i.e. immune evasion) cannot be functionally tested in this system.

At the same time, an ever-increasing number of parasite helminth genes are being proposed as potential immune evasion factors (29), having been identified by genomics, transcriptomics and classical biochemistry. Most current candidates as immune evasion molecules are those which, by sequence similarity, are recognizable homologues of known families of cytokines, enzymes or enzyme inhibitors. For example, in the case of *B. malayi* (30), homologues of the cytokines TGF- β (31,32) and macrophage migration inhibitory factor (MIF) (33–35) are likely to be involved in immunomodulation of the host response, while protease inhibitors of the cystatin (36) and serpin (37,38) families have also been postulated to interfere with specific pathways in certain host cell types. In many other species, such as

Fasciola hepatica (39) and *S. mansoni* (40), similar suites of parasite mediators are being actively identified. In nearly every instance, however, analysis depends on expression and purification of recombinant proteins for application to *in vitro* or *in vivo* assays. Such studies would be both enriched in scope and protected from problems of protein contamination, where transgenic expression of target genes possible.

It is also important to acknowledge that while some parasite immunomodulators will be recognizable by similarity to known genes, it is likely that the majority will be novel in sequence terms. We have already noted from a study on *N. brasiliensis* that within the subset of parasite genes bearing signal sequences (and which will include most of the secreted proteins from parasites), there is a substantially higher proportion of novel genes with no homologues in the database (41). One interpretation of this finding is that proteins secreted by parasites which interact with the host immune system will have evolved rapidly beyond similarity with known proteins from, for example, *C. elegans*, and that the rate of evolution may even be accelerated by an ‘arms race’ between host immune system genes and parasite immunomodulators (42).

For this reason, we considered whether more distantly related organisms could be transfected with helminth genes, and be tested in the setting of the host immune response. The criteria for a suitable test organism were first that the immune response to unmanipulated pathogens was well characterized, so that perturbations brought about by transgene expression could be readily detected; second, that it established a long-term chronic infection in which quantitative shifts resulting from transgene expression would be evident; and third, that transgenesis was a robust experimental system which express high levels of exogenous transgenes.

Each of these criteria was best matched by the protozoan parasite *Leishmania*. The immunology of infection with *Leishmania* species is exceptionally well documented (43,44), and in most combinations of parasite and host, this infection endures for many weeks as lesions grow and either are resolved or lead to fatality. In the case of *Leishmania mexicana*, an

additional advantage is its ability to productively infect macrophages *in vitro*, providing together with *in vivo* infection, two independent sets of immunological assays for each transgenic construct.

Moreover, transfection is reliable and straightforward (45). In this system, homologous recombination occurs with the SSU locus (of which there are numerous copies), so that integrants are stable. Transfection is performed on the promastigote stage, which can be cultivated *in vitro* as an extracellular form, dividing rapidly and allowing multiple lines to be isolated. The *in vitro* phase also permits the growth rate and viability of lines to be assessed, in case transgene expression exerts a deleterious physiological effect on the transfected organism.

TRANSFECTION APPROACH

We elected to use a design which integrates transgenes into the rRNA locus of *Leishmania* species (45). This uses the pSSU plasmid, which contains a multiple cloning site, allowing a choice of restriction sites depending on the insert sequence; the plasmid also contains ampicillin and puromycin resistance genes. Unless contraindicated, we have used *Xba*I and *Xho*I at the 5' and 3' ends, respectively, of a *Pfu*-amplified PCR fragment containing the entire open reading frame, including the start methionine and (where relevant) the endogenous signal sequence. Recombinant plasmid is restricted and ligated into pSSU, and the ligation product is transformed into *Escherichia coli*. Following verification of insert sequence, plasmid DNA is purified and linearized with *Pme*I, in preparation for transfection of promastigote parasites by electroporation. After 24 h, puromycin selection for positive integrants commences, and positive lines are isolated 2–3 weeks later. Each line is checked by PCR using plasmid and insert primers, on both DNA (to demonstrate transgene integration) and RNA (to ascertain expression, which is dependent upon integration into the intended SSU site). In practice, most, but not all, integrants are positive for both reactions. Finally, transgenic protein expression is verified by western blotting and immunofluorescence.

Multiple parasite lines can be isolated for each transfection, and are first tested for *in vitro* growth characteristics in comparison to wild-type parasites. In a number of different experiments with a range of transgenes, we have not yet observed a construct which exerts a deleterious effect on parasite growth. Lines of *in vitro*-propagated promastigote parasites are then tested for protein expression using antibodies specific for the transgene products by immunofluorescence.

Parasites can then be used both to infect macrophages *in vitro* and to infect mice, subcutaneously. Bone marrow-derived macrophages, grown from murine bone marrow for 7 days in macrophage-colony stimulating factor (M-CSF), are readily

infected by *L. mexicana*, and will, over the course of a further 7 days, harbour an increasing number of parasites. In this *in vitro* setting, the multiplication of intracellular parasites is measured by immunofluorescent microscopy on fixed cover slips used for culture of infected cells. In mice, *L. mexicana* establishes a chronic infection in most mouse strains, and lesion size is measured by micrometer in terms of footpad swelling following infection.

ABUNDANT LARVAL TRANSCRIPT (ALT) GENES OF *BRUGIA MALAYI*

We tested, as proof-of-principle, whether heterologous expression could shed light on the role of two highly abundant filarial genes which have no homologues of known function. These genes together make up ~5% of the cDNA of the infective larval stage of the parasite (46), and were named abundant larval transcript-1 and -2 (*ALT-1* and *ALT-2*). *ALT* has since been found to be a multigene family in filarial nematodes, and in the case of *B. malayi*, the two proteins *ALT-1* and *ALT-2* are ~80% identical (46,47). The proteins are relatively small (125–128 aa), containing a short acidic domain, variable between homologues, and a conserved domain extending over the C-terminal ~100 aa. A distantly related sequence exists in *C. elegans*, which lacks the acidic domain. We postulated therefore that the *ALT* proteins may be produced to allow the invading larvae to down-modulate the host immune system, and that such activity may be dependent on the acidic domain absent from the free-living organism.

Interest in the *ALT* proteins is also heightened by their particular promise as vaccine candidates for filarial infections. For example, gerbils immunized with *Bm-ALT-1* showed 76% protection against challenge (47), a finding corroborated by other laboratories working both with *B. malayi* (48,49) and *O. volvulus* (50,51).

Leishmania mexicana promastigotes transfected with either *ALT* homologue showed normal growth and viability (52). When probed by immunofluorescence with anti-*ALT* antibodies, transfectants were not only positive but also showed distinct staining along the flagella, indicating that the protozoa had successfully exported the signal-sequence-bearing *ALT* proteins to the plasma membrane. The same transfectants were also competent at macrophage infection, and indeed over a 7-day culture period were able to reach threefold higher levels of infection (parasites/macrophage) than the wild-type controls (Table 1).

A similar increase in infectivity was observed *in vivo*. Over the course of 12–16 weeks, *L. mexicana* establishes a chronic infection which slowly resolves. Mice infected with either *ALT* transfectant showed larger lesions, and higher residual parasite numbers, than those exposed to the wild-type parasite (52).

One of the advantages of transgenic systems is the facility to design and test mutant proteins. We therefore made a construct of ALT-2 which lacked the acidic domain [acidic domain deleted (ADD)]. *Leishmania mexicana* transfectants expressing ADD displayed the wild-type phenotype, demonstrating that the acidic domain was essential for the modulatory properties of ALT-2 (52).

Finally, the ability of *L. mexicana* to infect macrophages *in vitro* allowed us to test, by a small gene array, the spectrum of gene expression in cells infected with wild-type or ALT-transfected parasites. In replicate experiments, two genes were most markedly up-regulated in cells containing either ALT-1- or ALT-2-expressing organisms. These were SOCS-1, a negative regulator of IFN- γ signalling; and GATA-3, a key transcription factor which promotes Th2 differentiation. The biological relevance of these observations was enhanced by showing that mice exposed to live infective larvae of *B. malayi* (the stage expressing high amounts of ALT-1 and -2) up-regulated SOCS-1 and GATA-3 *in vivo* (52). In this way, the *Leishmania* transfection system has revealed a hitherto unsuspected interaction between proteins secreted from the extracellular helminth parasite and intracellular signalling proteins of the host immune system.

Cysteine protease inhibitor (CPI-2)

Filarial cysteine protease inhibitors represent a prominent part of the antigenic and immunological profile of the parasite (53). CPI-2 is a 15 kDa surface and secreted protein with sequence similarity to the cystatin family of protease inhibitors. *Bm*-CPI-2 contains two inhibitory sites, which, respectively, block classical cysteine proteases and the more recently discovered asparaginyl endopeptidase (AEP) family (54). Inhibition of AEP is thought to be responsible for the ability of *Bm*-CPI-2 to block MHC class II-dependent antigen processing in human cells (54). The AEP-inhibiting site is found only in filarial, and in mammalian, members of the family, and is absent from *C. elegans* homologues, and from other known invertebrate sequences. Site-directed mutagenesis analysis has shown that the same residues are required for AEP inhibition in both mammalian and filarial cystatins, indicating a precise degree of convergent molecular evolution may have occurred to match the parasite inhibitor to the target host protease (36). Further evidence of immunomodulation by cystatins has been found from other filarial parasites such as *Onchocerca* and *Acanthocheilonema* (55,56).

A transfectant of *L. mexicana* expressing *Bm*-CPI-2 was constructed to test the hypothesis that *Bm*-CPI-2 interferes with class II antigen processing *in vivo*, and might therefore, if expressed in *Leishmania*-infected macrophages, reduce stimulation of a protective immune response. As expected, there was no alteration in growth or infection of macrophages

in vitro. However, the *in vivo* dynamics of this transfectant were also similar to the wild-type, and hence in the context of this experimental test, CPI-2 was found not to be immunomodulatory.

Macrophage migration inhibitory factor (MIF)

One of the more remarkable findings to emerge from analysis of nematode gene sequences has been the presence of homologues of the mammalian cytokine MIF (33,35). In particular, the secretion of parasite-derived MIF was surprising in view of the assignation of the mammalian protein as a proinflammatory cytokine; however, repeated *in vivo* administration of *Bm*-MIF-1 to mice had shown that it induced macrophages to produce Ym-1, a specific marker of macrophages that had entered a state of alternative activation, rather than classical proinflammatory activation (34).

We reasoned, further, that MIF may play different roles in various contexts, especially as evidence from the mammalian system was that its effects were highly dose-dependent. Hence, it was possible that continuous exposure of immune cells to MIF may in fact be counter-inflammatory. To explore these concepts, we constructed *B. malayi*-MIF transgenic *L. mexicana* (*L. Prieto-Lafuente*, unpublished observation). In the initial round of experiments, MIF-expressing *L. mexicana* promastigotes were found to grow at the same rate as wild-type, but the transgenic parasites fared poorly in macrophages. Despite similar initial infection rates, few macrophages harboured more than two transgenic *L. mexicana* organisms per cell, and overall rates were threefold lower than wild-type.

Surprisingly, then, we found that *in vivo* the MIF-expressing *L. mexicana* survived as well, if not better, than wild-type parasites when assessed at 4 or 8 weeks post-infection. However, when taken *ex vivo*, the same transgenic parasites retained their poor *in vitro* growth capacity. Hence, survival *in vivo* was not due to inactivation of the transgene, and could more likely be attributed to the failure of continuous MIF to induce an inflammatory response.

Venom allergen/activation on secretion-like protein (VAL)

It is striking that in almost every helminth parasite species so far studied, members of one particular gene family are prominently expressed. The first nematode homologue discovered was a protein secreted by larval *Ancylostoma caninum* (ASP), which has developed into a major vaccine antigen for hookworm. The family has extended to include plant pathogenesis-related proteins (PrPs) and mammalian sperm cysteine-rich secretory proteins (CRISPs), which has been associated both with calcium flux regulation (57) and with mediating sperm binding to the egg (58). In insects, the

family is typified by the venom allergens such as Ves v5, or antigen 5 (59), giving rise to an alternative acronym, CAP [crisp, antigen 5, Pr-1: (57)]. Related proteins in both *Xenopus* and in lizards are also thought to control calcium signalling (57). In *B. malayi*, one member of this family (*Bm-VAL-1*) is the second most abundantly expressed gene after *alt-2*, and is known to be strongly immunostimulatory in animals and in humans (60). Similar proteins have been found at early stages of small expressed sequence tag (EST) sequencing projects in *H. contortus* (61), *N. brasiliensis* (41), *Ostertagia ostertagi* (62) *St. stercoralis* (63) and *Toxocara canis* (64).

Because functional studies in insects, plants and vertebrates are both limited and disparate, it is difficult to formulate a hypothesis about the role of VALs in the helminth–host relationship. Moreover, the very wide distribution of this gene family could indicate that it represents a flexible structural framework for proteins which have evolved quite different functions in very different settings. Hence, the study of VAL protein function is very similar to that of ALTs in that there should be a minimum of preconception about its potential role. For these reasons, we have also studied VAL transfection in the *Leishmania* system.

Recently, we have isolated *Bm-VAL-1*-transgenic *L. mexicana* and shown VAL-1 expression in the promastigote (extracellular) stage both by RT-PCR and by immunofluorescence with a VAL-1-specific monoclonal antibody (J. Murray *et al.* unpublished observation). As with ALT transfectants, staining extended to the flagella of the promastigote, indicating that the *Brugia* gene product had been exported to the plasma membrane of the parasite. Furthermore, macrophages infected with the transgenic parasites were also stained by the anti-VAL monoclonal antibody, and culture supernatants from infected macrophages reacted by Western blot with the same antibody. Hence, there is release from the host cell of a protein encoded by a transgene within the intracellular parasite. Experiments are now under way to assess the impact of such expression on the course of infection *in vivo*.

FURTHER DEVELOPMENT OF THE METHOD

The studies outlined above have shown that heterologous expression of helminth genes in *Leishmania* can be used to test immunological function both in the specific *in vitro* context of macrophages and also in the broader *in vivo* setting. The results also justify further elaboration of the approach. For example, incorporation of different cell trafficking signals might test whether products such as ALT are directed to targeted compartments and/or are able to cross host cell membranes. Because the MIF proteins are unusual in being secreted without a signal sequence, similar localization

studies would be particularly interesting. In general, it would be appropriate to address the issue of how transgene products are exported into the macrophage cytoplasm and if they are further released into the extracellular environment of the host.

A further application of this experimental system will be extension into transfection of *L. major*, which can be achieved with exactly the same plasmid system. Although *L. major* does not readily infect macrophages *in vitro*, and requires prompt *in vivo* passage for propagation (a constraint on the selection of transfected lines), the different patterns of resistance and susceptibility in BALB/c and C57BL/6 mice would allow a clear question to be asked: does ALT expression switch the host response of a resistant C57BL/6 mouse to the Th2 (susceptible, BALB/c-like) phenotype?

In conclusion, functional genomics with helminth pathogens will continue to require an imaginative combination of approaches, and considerable further innovation. The host organism selected for transfection may vary considerably according to the underlying scientific question. For example, in researching the ability of *Trichinella spiralis* to reprogramme myocyte gene expression, Jasmer and colleagues used a mammalian expression plasmid containing *T. spiralis* p43 to transfect a skeletal muscle cell line *in vitro* (65). Similarly, the recent demonstration that parasite enzymes, which may be intractable to functional expression in prokaryotic systems, can successfully be expressed in *C. elegans* allows their purification in active form (66). The combination of this spectrum of mammalian, protozoan and nematode expression systems illustrates the expanding platform which will be required for future analysis of gene function in the major helminth pathogens of humans and animals.

ACKNOWLEDGEMENTS

The authors acknowledge support from the Wellcome Trust (through Programme Grant support to R.M.M., a Henry Wellcome ‘Showcase’ Grant and a Project Grant (support for L.P.L.). We thank Clare Blackburn, Yvonne Harcus, Clare Bennett and Lisa Colledge for many invaluable contributions to this project.

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