From genomes to vaccines: *Leishmania* as a model

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The 35 Mb genome of *Leishmania* should be sequenced by late 2002. It contains approximately 8500 genes that will probably translate into more than 10 000 proteins. In the laboratory we have been piloting strategies to try to harness the power of the genome–proteome for rapid screening of new vaccine candidate. To this end, microarray analysis of 1094 unique genes identified using an EST analysis of 2091 cDNA clones from spliced leader libraries prepared from different developmental stages of *Leishmania* has been employed. The plan was to identify amastigote-expressed genes that could be used in high-throughput DNA-vaccine screens to identify potential new vaccine candidates. Despite the lack of transcriptional regulation that polycistronic transcription in *Leishmania* dictates, the data provide evidence for a high level of post-transcriptional regulation of RNA abundance during the developmental cycle of promastigotes in culture and in lesion-derived amastigotes of *Leishmania major*. This has provided 147 candidates from the 1094 unique genes that are specifically upregulated in amastigotes and are being used in vaccine studies. Using DNA vaccination, it was demonstrated that pooling strategies can work to identify protective vaccines, but it was found that some potentially protective antigens are masked by other disease-exacerbatory antigens in the pool. A total of 100 new vaccine candidates are currently being tested separately and in pools to extend this analysis, and to facilitate retrospective bioinformatic analysis to develop predictive algorithms for sequences that constitute potentially protective antigens. We are also working with other members of the *Leishmania* Genome Network to determine whether RNA expression determined by microarray analyses parallels expression at the protein level. We believe we are making good progress in developing strategies that will allow rapid translation of the sequence of *Leishmania* into potential interventions for disease control in humans.

Keywords: *Leishmania*; genome; vaccines

1. INTRODUCTION

Leishmaniasis remains a major public health problem throughout the tropics. The current outbreak of kala-azar or visceral leishmaniasis caused by *Leishmania donovani sensu strictu* in eastern and southern Sudan has taken its toll on an impoverished, war-stricken population, killing tens of thousands and depopulating vast areas of southern Sudan. The calamity brought kala-azar to the forefront as one of the greatest epidemics of the 20th century (Perea *et al.* 1989; De Beer *et al.* 1990). Post kala-azar dermal leishmaniasis following drug treatment causes additional problems, as do the dramatic and debilitating cutaneous forms of disease that occur throughout tropical regions of both the Old and New Worlds. Even in sub-tropical regions where disease has not traditionally caused a major problem in humans, sub-clinical status is elevated to clinical disease in association with HIV infection (Rosenthal

et al. 2000). There are no vaccines in routine use, and chemotherapy still relies principally on antimonial-based drugs first used in the early 20th century.

The 35 Mb genome of *Leishmania* should be sequenced by late 2002. It contains approximately 8500 genes that it is assumed will translate into more than 10 000 proteins. The question is: how rapidly can we harness the power of the genome–proteome to learn more about the biology of the host–parasite interaction and to develop new drugs and new vaccines? In our laboratory we have focussed on DNA-vaccine technology to develop higher throughput screens for potential new vaccine candidates. Initially the following two questions were considered.

- (i) Could we narrow down the number of genes to be tested as vaccines by prescreening for genes expressed in forms of the parasite that invade and survive in the vertebrate host?
- (ii) Could we test DNA vaccines in pools?

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Figure 1. Results of partition-based clustering of expression profiles for one experiment in which RNAs from different points in the development cycle (days 3, 5, 7, 10 for promastigotes grown in Schneider's insect medium) for *L. major* LV39. See § 2 for further description of the analysis.

2. EXPRESSION PROFILING USING cDNA MICROARRAYS

The analysis of gene expression at the RNA level using microarray techniques can provide a global genetic perspective on biological processes important in parasite survival and host-parasite interactions. In *Leishmania*, most gene transcription is polycistronic. Transcript abundance measured using microarray techniques will therefore reflect post-transcriptional regulation of gene expression through processes including regulation of mRNA stability. The extent to which transcript abundance reflects ex-

pression at the protein level has yet to be determined. The first step was to determine whether significant differences in transcript abundance could be measured at the RNA level using microarray techniques.

In the sandfly vector, *Leishmania* undergoes developmental changes that lead to the generation of infective metacyclic forms transmitted to the vertebrate host. This can be mimicked in culture as promastigote forms of the parasite pass through logarithmic to stationary phases of the growth cycle. To study the developmental changes in transcript abundance, total RNA was prepared from

promastigotes at days 3, 5, 7, 10 and 13 of culture and from LAs for *L. major* LV39.

As a pilot microarrays were produced by spotting PCR-amplified inserts from 2091 randomly selected full-length cDNAs from spliced leader libraries prepared from different developmental stages (days 3, 7 and 10 cultured promastigotes *L. major* LV39; LAs from *L. major* Friedlin) (Coulson *et al.* 1996; Levick *et al.* 1996). These clones had already been used for EST one-pass 5' sequence analysis (Levick *et al.* 1996; R. Almeida, A. Norrish and J. M. Blackwell, unpublished data). A complete list of the EST sequences, and the cDNA libraries from which they were derived, can be obtained at <http://www.ebi.ac.uk/parasites/leish.html>. Sequence-cluster analysis shows that the 2091 genes represent 1094 unique transcripts, that is, there was around 50% redundancy in the clones selected for random-sequence analysis. For the clustering, a BLASTN analysis of all *L. major* ESTs against the original set (Levick *et al.* 1996) in the dbEST database was performed. All hits with probabilities $<1e^{-10}$ were initially ranked in descending order of quality. Sequences were then placed in 1094 'pools' (some contain only one entry) on the basis of these data. *Leishmania* sequences with potentially interesting BLAST similarities to constituents of the non-redundant nucleotide, dbEST or Swissprot protein sequence databases comprised 30% of the total EST dataset. These hits represent the 'top' non-self hit obtained for each sequence (the data have been parsed: probability cut-off of $1e^{-05}$). Out of these only 23% have significant matches to genes of known functions such that they can be assigned a putative function. Seventy percent of the clones have no sequence identity to any other sequences in the public databases. The 1094 unique genes represent approximately 12% of the total estimated number of *Leishmania* genes.

To screen the microarrays, a single round of reverse transcription was used to tag RNA transcripts for a particular developmental stage with Cy3. To compare changes in transcript abundance across different developmental stages tested on different microarray 'chips' we had to decide on a control RNA that could be labelled with Cy5 and competitively hybridized with Cy3-labelled 'test' cDNA on every chip. In the initial experiment looking for genes upregulated in expression in amastigotes (figure 1), control RNA was used from a single time-point: day 5 cultured promastigotes of *L. donovani* LV9. More recently, a pool of RNA across different developmental stages was used for a single outgroup 'control' species; for example, a pool of RNA from actively growing cultures from days 3, 5 and 7 for *L. amazonensis* has been used to compare changes in transcript abundance for different developmental stages across *L. donovani* and *L. major*. Full details of the results of these experiments will be presented elsewhere (Almeida *et al.*, unpublished data). All of the data will be deposited in the public domain (<http://www.ebi.ac.uk/parasites/leish.html>) with links that will allow the data to be re-interrogated online at the European Bioinformatics Institute (<http://www.ep.ebi.ac.uk/EP/EPCLUST/>). To compare changes in transcript abundance across developmental stages the data were analysed using both hierarchical and partition-based clustering algorithms (Brazma & Vilo 2000) (<http://industry.ebi.ac.uk/~vilo/Expression-Profiler/>). To understand how the

Figure 2. Scatter plot showing fluorescence intensity on the *x*- and *y*-axes for Cy5-labelled control RNA from the control pool against Cy3-labelled RNA from *L. major* amastigotes. The *x*- and *y*-axes have already been adjusted to eliminate background fluorescence, so every spot on the graph is expressed above the background. The individual points on the scatter plot represent the 2091 independent clones on the microarrays. The dotted lines above and below the ratio 1:1 line indicate the cut-offs for two-fold upregulation or two-fold downregulation relative to the control pool. Two dots are annotated to demonstrate that two clones may have the same absolute fluorescence intensity on the Cy3 amastigote axis, but can be $>+2$ -fold and <-2 -fold relative to the control pool.

clustering programs make such comparisons, it is useful initially to look at a scatter plot for Cy3 against Cy5 spot intensities for a single developmental stage within a single experiment. Since we want to identify amastigote-expressed genes for potential vaccine development, the scatter plot for RNA harvested from LA of *L. major* LV39 (Cy3) against the *L. amazonensis* RNA pool (Cy5) is of direct relevance (figure 2). Each spot on the scatter plot represents a single cDNA clone. To declare a significant change in transcript abundance between the developmental stages, the ratio of intensity of the Cy3 'test' RNA : Cy5 'control' RNA needs to be greater than +2 or less than -2, that is, there needs to be more than a two-fold enhancement or reduction in transcript abundance comparing 'test' and 'control' RNAs. On the scatter plot this includes the spots above and below the dotted lines. It should be noted, however, that two different spots may have the same absolute intensity for Cy3 (on the *y*-axis), but one can show more than two-fold enhancement (above the +2-fold dotted line) while the other can show a more than two-fold reduction in intensity compared to their respective Cy5 controls. This will be reflected differently in the patterns of changes in transcript abundance that the cluster analysis will portray (cf. below). Spots that fall within the two dotted lines do not differ significantly in intensity from the control pool, and will cluster around the 'zero' change line on the cluster analysis plots. Again, many of the genes falling within this region will have high absolute intensity on the Cy3 axis. Hence, if the question is simply to ask which genes are expressed in amastigotes, we might choose to set a cut-off on the Cy3 axis above which all genes (= spots) will be considered to be

Table 1. Identity of clones from clusters of genes upregulated in amastigotes of *L. major* relative to the day 5 promastigote control. ((a) The cluster depicted in figure 1e contains all the redundant copies of the tryparedoxin peroxidase gene; (b) the cluster depicted in figure 1k contains six out of seven redundant clones of the nucleoside diphosphate kinase B gene. The output of EPCLUST provides the clone name, accession number, and top matches and scores from BLAST analyses, permitting identification of clones coregulated with these genes.)

(a) tryparedoxin peroxidase cluster

6381 AA728310 32 5.90E-12 TR:O61000 *Crithidia fasciculata* tryparedoxin peroxidase
lmf030 T67356 32 1.40E-16 TR:O96763 *Trypanosoma cruzi* tryparedoxin peroxidase
lmh018 H64196 32 3.80E-40 TR:O61000 *C. fasciculata* tryparedoxin peroxidase
lmf030 T67356 32 1.40E-16 TR:O96763 *T. cruzi* tryparedoxin peroxidase
k085 AA680724 32 3.00E45 SW:TDX TRYBR *T. brucei* thioredoxin peroxidase

lm370 T93440 145 1.10E-65 SW:METK_LEIIN *Leishmania infantum* S-adenosylmethionine synthetase
lmk508 W88371 56 2.00E-32 TR:Q9N6Y7 *L. major* probable nucleoside diphosphate kinase B
021c A1034563 119 1.80E-27 TR:O46368 *Leishmania mexicana* surface protein amastin
s033 AA680974 55 1.10E-25 SW:IF51 SCHPO initiation factor EIF-5A-l
mb017 T67314 294 3.90E-11 SW:H2A PLAF A *P. falciparum* histone H2A
202c A1034553 294 7.70E-29 SW:H2A2 LEIDO *Leishmania donovani* histone H2A.2
lmk414 W88357 218 6.50E-31 TR:Q9N9A8 *L. major* possible ubiquitin fusion protein
5151 AA728269 342 1.10E-44 TR:P90633 *Leishmania braziliensis* elongation factor EF-1A
581m AA728297 187 3.40E-54 TR:Q9NE87 *L. major* hypothetical L3640.07
lmk271 W88395 162 2.90E-21 TR:Q9W3W8 *Drosophila melanogaster* CG3203 protein
lm393 T93450 311 3.50E-55 TR:O02498 *L. major* RPS7
362d A1034646 276 6.10E-20 TR:O97213 *L. major* L4830.9 protein

14 × 60S ribosomal proteins

13 × 40S ribosomal proteins

9 × other ribosomal proteins

66 × unknowns including lmd029 T67335 260 n.s. (1.0e – 10)

(b) nucleoside diphosphate kinase B cluster

lmk410 AA060749 56 2.20E-57 TR:Q9N6Y7 *L. major* nucleoside diphosphate kinase B
168c A1034527 56 1.30E-47 TR:Q9N6Y7 *L. major* nucleoside diphosphate kinase B
lmh014 T67339 56 7.70E-57 TR:Q9N6Y7 *L. major* nucleoside diphosphate kinase B
664c A1034834 56 6.60E-103 TR:Q9U1E1 *L. major* nucleoside diphosphate kinase B
q072 AA680908 56 4.40E-70 TR:Q9N6Y7 *L. major* nucleoside diphosphate kinase B
6531 AA728315 56 2.30E-26 TR:Q9N6Y7 *L. major* nucleoside diphosphate kinase B

438c M034692 82 2.20E-71 TR:Q9NK14 *L. major* mitotic centromere associated kinesin 2
214a AA741623 92 1.10E-25 SW:H4 CHLRE *Chlamydomonas reinhardtii* histone H4
292d A1034608 92 1.60E-17 TR:O62586 *Blespharisma undulans* histone H4
6831 AA728322 92 7.70E-13 TR:Q9U9A5 *Leishmania tarentolae* histone H4

4 × 60S ribosomal proteins

1 × other ribosomal proteins

7 × unknowns

expressed in amastigotes. The stringency of the cut-off above this can be determined according to user requirements; for example, in the initial targeting of amastigote-expressed genes as vaccine candidates we might choose to set a cut-off a log-fold above the background. However, since we cannot be sure that transcript abundance relates directly to protein abundance, it might be prudent to sample and test genes with both high- and low-transcript abundance.

To obtain an overall profile of the changes in transcript abundance for each gene throughout the developmental cycle we first used partition-based clustering asking the clustering algorithm to cluster our 2091 genes around 10 'seed' clones that it chose as representative of the range of different expression profiles. Figure 1 shows this analysis for the experiment in which the different developmen-

tal stages for *L. major* LV39 were each compared against day 5 *L. donovani* promastigote RNA. The 10 seeds are shown in figure 1j. The program iteratively compares each of the seeds to its next closest matching expression profile, recalculating the cluster means or centres as it goes. The final cluster centres are shown in figure 1l. The 10 resulting clusters are shown in figure 1a–i, k. The profiles show log₂ ratios for fold-increases above and below the control RNA (log₂ of the ratio 1 = 0). The biggest change in expression relative to control for any genes within a cluster are from log₂ = –1 to log₂ = +4 (figure 1b), that translates to a 32-fold difference in relative abundance for particular genes within the cluster between day 5 promastigotes and amastigotes. The clusters in figure 1b,e,h,k are of particular interest because they contain clusters of genes that appear to be strongly upregulated in abundance in

Figure 3. Key results from the DNA-vaccine experiments. (a) Typical results for lesion growth phenotypes in susceptible BALB/c versus resistant CBA/Ca mice; (b) s.c. vaccination with $1 \times 25 \mu\text{g}$ lmd29 versus vector control; (c) results for vaccination with pools (P1–P8) versus lmd29 and vector control, where P3 and P8 are significantly protected compared with the vector; (d) the effect of vaccination with LmaR61, the DNA vaccine at the intersect of P3 and P8.

the amastigote stage of the parasite life cycle, and hence might be good vaccine candidates. Looking within these clusters, it can be seen that all redundant clones of the trypanodioxin peroxidase gene fall within cluster 1e (table 1a), while six out of seven redundant clones of the nucleoside diphosphate kinase B gene fall within cluster 1i (table 1b). This is reassuring, since it demonstrates remarkable within-experiment reproducibility that extends over all redundant sets of clones within all of our experiments (data not shown). We can then begin to ask questions about what other genes that might be related in function show parallel expression profiles. For example, genes coregulated with nucleoside diphosphate kinase B include histone 4 and the mitotic centromere associated kinensin 2 (table 1b). This is of interest given that highly conserved homologues of nucleoside diphosphate kinase B occur across prokaryote and eukaryote organisms. It is a dual-action enzyme that maintains the intracellular level of 5'-nucleoside triphosphates (Gilles *et al.* 1991) and also acts to regulate transcription (Postel *et al.* 1993). In *Drosophila* it is known to regulate cellular differentiation and development (de la Rosa *et al.* 1995). It also functions to regulate transcription by the phosphorylation of transcription fac-

tors in a DNA-dependent manner (Lees-Miller 1996). The enzyme is upregulated in *Trypanosoma brucei* during the S phase of the cell cycle (Gale *et al.* 1994), indicating that it plays some role in the cell cycle in trypanosomatids. A more eclectic set of genes cluster with trypanodioxin peroxidase (table 1a), frustratingly including 66 genes of unknown function that could relate to the antioxidant pathway in which this gene plays a central role (Levick *et al.* 1998; Tetaud & Fairlamb 1998; Alphey *et al.* 2000). A more in-depth analysis of this cluster of unknown genes, perhaps lowering the stringent cut-off originally set to declare significant sequence identity, might reveal genes of related function and importance. Experiments could also be performed to determine which of these genes responds to changes in experimentally induced oxidative stress, thus helping to delimit which genes might contribute to antioxidant pathways.

3. FROM GENES TO VACCINES

The most direct route to taking our putative amastigote-expressed genes into vaccine testing was to employ a DNA-vaccine strategy. DNA vaccination has already

proved successful in vaccinating mice against cutaneous *L. major* infection (Gurunathan *et al.* 1997, 1998; Handman *et al.* 2000; Fragaki *et al.* 2001). A number of experiments (Norrish *et al.*, unpublished data) have been carried out in our laboratory to refine the DNA-vaccine strategy, in particular to see whether it was possible to reduce both the number and dose of vaccines given. This would facilitate a higher throughput of large numbers of potential vaccine candidates in a murine screen, and allow us to think about pooling strategies to try to identify new vaccine candidates more efficiently. Figure 3 shows the results of one such experiment. Figure 3*a* shows the normal profile for *L. major* LV39 infection in susceptible BALB/c compared with genetically resistant CBA/Ca mice. Figure 3*b* shows that vaccination with 100 µg vector DNA alone mimics the BALB/c infection-only profile, whereas vaccination with a single dose of 25 µg DNA of a protective antigen lmd29 (plus 75 µg vector-only DNA) administered s.c. 6 weeks prior to challenge infection provides significant protection. Figure 3*c* depicts the infection profiles for eight pools of 4 × 25 µg DNA for 16 unknown test vaccines from a 4 × 4 array. Two vaccine pools show some protection relative to the vector-only control; the remaining six pools gave the same infection profile, or showed exacerbation of disease, relative to the vector-only control. Figure 3*d* shows that the vaccine at the intersect of the two protective pools, designated R61, is protective when administered on its own. This strategy was therefore successful in identifying a new vaccine candidate from the 16 test vaccines in the pools. However, it was also found that protection was lost when 25 µg lmd29 was administered in a pool with 25 µg each for three members of one of the non-protective pools (data not shown). Hence, there may be antigens within pools that exacerbate disease and mask the potential protective effect of other members of the pool. In current experiments this analysis is being extended by testing the 20 pools obtained in a 10 × 10 array, alongside each of the 100 vaccine candidates tested separately. One advantage of testing all the vaccine candidates separately is that, with the full sequence of all the vaccine candidates to hand, it will be possible to perform a retrospective bioinformatic analysis to see if a predictive algorithm can be devised for what constitutes a successful vaccine candidate for leishmaniasis.

4. CONCLUSIONS

In the laboratory we have been piloting experiments designed to see whether it is possible to develop global approaches to identify amastigote-expressed genes and carry out large-scale vaccine testing directly utilizing the output of the genome project. The findings thus far have been encouraging, but there is much work ahead to harness the full power of the genome–proteome. In relation to microarray analysis, it is necessary to determine whether expression profiling at the RNA level bears any relation to expression of the protein encoded by the RNA. To examine this we are working with other members of the *Leishmania* Genome Network to carry out parallel analyses of developmental changes in the transcriptome and the proteome using material harvested from the same cultures of parasites. We are also taking some of our protective antigens to see if we can improve vaccine efficiency using a

DNA-vaccine prime and protein boost strategy (McShane *et al.* 2001) employing attenuated *Salmonella typhimurium* or *Vaccinia* virus as the protein-producing vaccine. Clearly the real challenge will be to see how rapidly we can proceed from animal experimentation to phase I and II trials in humans, thus proving the real worth that knowledge of parasite genomes will probably prove to be in relation to human health.

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