Report on Leishmaniasis
Report of the Scientific Working Group
meeting on Leishmaniasis
Geneva, 2–4 February, 2004
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1. Rationale for convening the Scientific Working Group on Leishmaniasis

The goal of the Scientific Working Group (SWG) meeting was to identify leishmaniasis research priorities for the next five years. In addition, the group was to identify strategic emphases, based on TDR’s comparative advantage, to add to the current ones. The discussions were to be focused on how to cope with and prevent drug resistance, and how to optimize the use of genomic information to improve drug, diagnostics and vaccine discovery. Additionally, the group was requested to consider if leishmaniasis should remain on the list of TDR category I diseases (emerging and uncontrolled; worsening epidemiological situation; increasing incidence of infection and disease).

The meeting provided an opportunity to discuss the tools needed to control leishmaniasis, i.e. diagnostics, drugs, vector control, and vaccines, and to assess their adequacy as well as the new knowledge and new tools needed to improve the current tools and control measures.

The SWG reiterated that control of leishmaniasis is achievable, although current tools have proved insufficient for dealing with the biological complexity of the disease and with the unmanageable changes caused by environmental, societal, political and behavioural factors.

Currently available tools need to be improved and validated under diverse field realities. One of the best drugs available on the market for treatment of visceral leishmaniasis, Ambisome, is inaccessible to populations in need due to its high cost. Methods must be sought to advocate for reduced prices for such drugs. This highlights the inequities of access to available tools and healthcare, which plague people suffering from leishmaniasis. Despite the high effectiveness of some of the current drugs, development of new drugs must be pursued in face of the constant threat of resistance and the clear limitations of available drugs.

Preventing disease morbidity and mortality remains of prime strategic importance; this requires vector control, including evaluating behaviour changes and susceptibility to insecticides, as well as better and affordable diagnostics for early case detection coupled with adequate treatment. These measures constitute a key mechanism for limiting the human reservoir in anthroponotic foci. Development of a vaccine against leishmaniasis also remains a prime strategic goal, which requires improved knowledge of pathogenesis of the disease.

The SWG concluded that, while visceral leishmaniasis (VL) should remain TDR’s highest priority, research into other forms of leishmaniasis is necessary both because they are diseases in their own right and in order to gain knowledge to aid in the fight against VL. Specific recommendations are given for the development of new and improved intervention
methods, prevention strategies and policies. The recommendations for generating needed basic knowledge and new tools are less specific and point to areas where gaps exist.

It is evident that the needs for research aimed at control of leishmaniasis are numerous and that TDR can benefit from working with various partners.
2. Recommendations

The SWG recommended that the following areas should receive special attention:

• Validation of existing tools for leishmaniasis control by a global network of clinical trial centres.
• Operational research to ensure effective implementation of control strategies.
• Use of innovative approaches including those generated from genomic information to improve upon existing and to develop new diagnostic, therapeutic and epidemiological tools as well as to develop vaccines.
• Research for better understanding of the pathogenesis and the mechanisms of protection against the human disease.
• Co-funding of research activities on leishmaniasis in cooperation with interested parties including those within endemic countries.
• Research capacity strengthening in all these research areas in disease endemic countries.
3. Introduction

Leishmaniasis is the collective name for a number of diseases caused by protozoan flagellates of the genus *Leishmania*, which have diverse clinical manifestations. The form that is deadly is visceral leishmaniasis (VL), caused by *Leishmania donovani* in the Old World and *L. infantum* in both the Old and New World. If left untreated, full-blown VL invariably leads to death, thus making early diagnosis of paramount importance. A number of different species of *Leishmania* cause cutaneous (CL) and mucocutaneous (MCL) leishmaniasis, which, if not fatal, are still responsible for considerable morbidity of a vast number of people in endemic foci. The estimated global burden of disease is believed to be inaccurate due in part to the passive case detection data used to estimate the disease prevalence in many endemic countries. The current global estimate of 350 million people in 88 countries at risk of leishmaniasis and of an incidence per year of 500,000 for VL and 1.5 million for CL belies higher burdens of disease in endemic foci.

A number of challenges exist for the prevention and control of leishmaniasis. Endemic leishmaniasis is perceived as a problem of low priority by governments, society, and in some instances by the patients themselves. Factors that contribute to this perception of low priority include:

- the leishmaniases are diseases of poor people in peripheral areas far from the centre of government
- other or urgent health problems are of greater magnitude or have greater impact
- resources are limited
- there is a lack of information about the current magnitude or nature of the problem
- there is no knowledge about the most effective points of intervention
- affected communities are ignorant about the importance and availability of diagnosis and treatment.

Epidemics of leishmaniasis are known to occur frequently in many foci, but often the strategy of government and health authorities is to react rather than to anticipate and prevent.

The leishmaniases are dynamic diseases and the circumstances of transmission are continually changing in relation to environmental, demographic and human behavioural factors. Changes in the habitat of the natural host and the vector, HIV infection, and the consequences of conflict, all contribute to the changing leishmaniasis landscape. Regular epidemiological studies to address risk factors and transmission patterns are necessary in order to integrate information on the current situation into control strategies.
The tools currently available for the control of leishmaniasis are limited in number and utility, and have not been completely validated. Hence intervention research is needed in order to validate the tools in the field, and new and improved tools are required. To achieve this, a better understanding of the human diseases is essential; this is best studied in human beings but would be facilitated by having improved animal models that more accurately reflect the human diseases.

Due to the dynamics of the disease and the substantial developments provided by ongoing innovative research in other disciplines, the recommendations presented in this document are not finite. An element of flexibility is needed in order to ensure that opportunities for advancing knowledge and developing means of controlling this disease are taken advantage of.

While TDR has the comparative advantage to take a number of research areas forward, the daunting need for more research requires that TDR partner with other organizations and with industry to achieve the recommendations set out in this report. Partners may include other funding agencies, national governments, and grassroots organizations found locally in disease endemic areas.

Epidemics occur, and we need to be ready for them. This involves being prepared with validated diagnostic tools as well as research results which aid the prediction of epidemics.

The following sections describe the rationale behind the SWG recommendations on research priorities, the appropriate utilization of available tools, and the development of new tools to reduce the burden of leishmaniasis.
4. Generating evidence to improve the effectiveness of control programmes

To maximize the likely impact on global burden of disease, the priority for applied research related to leishmaniasis control policy should be VL, the lethal form of leishmaniasis, but not to the exclusion of CL/MCL, which is a major health concern because of the higher morbidity of CL (localized and diffuse, DCL [diffuse cutaneous leishmaniasis]) and the debilitating nature of MCL. Visceral leishmaniasis is largely a disease of the rural poor, with the possible exception of Brazil where there is increasing evidence that zoonotic VL is becoming urbanized. Furthermore, domestic transmission is becoming widespread in settlements on previously underdeveloped lands, while outbreaks at the peripheries of urban centres in smaller cities have been reported in Colombia and Venezuela. A major reason for the continuing high burden of disease caused by VL worldwide is the relatively low proportion of patients who have access to, are provided with, or who complete, a full course of safe and efficacious drugs. The reasons for this are many, but include:

- the limited access to effective diagnostic tests where they are needed
- problems with the sensitivity and specificity of currently available diagnostic tests
- the limited availability of drugs for rural populations
- poor drug adherence due in part to the relatively long course of parenteral treatment generally required
- the frequently reported high toxicity of the principal first-line drugs in use worldwide (pentavalent antimonials [Sb])
- the development of widespread resistance to first-line drugs, and its spread from the epicentre of the VL endemic region in India
- the high cost of the best available second-line drug for treatment of VL (Ambisome).

Many of these problems need to be addressed by implementation research, including social and economic studies (see below), and by strong advocacy for increasing resources to improve access to drugs and reduce prices. Significant progress in reducing the burden of VL can also be made by the speedy but regulated introduction of new tools for diagnosing and treating patients.

In recent years, with the support of TDR and other agencies, new tools for diagnosis and treatment have been developed which should be urgently tested for effectiveness across all VL endemic regions; and new/improved diagnostic tools and drugs are likely to emerge in the near future. Standardized procedures and large sample sizes are vital to ensure that definitive conclusions can be drawn from such studies, leading to immediate evidence-based policy decisions. Hence, the formation of an extensive Leishmaniasis Clinical Trial Group (LCTG) for VL, CL/MCL and DCL – a network of WHO-certified centres across endemic regions for undertaking multicentric clinical trials of currently available and future drugs, diagnostics, and (as they become available) vaccines – is strongly recommended. Existing networks of clinical trial centres working on leishmaniasis should be further strengthened and consolidated into the LCTG. In relation to the currently available tools, the priority areas for research on diagnostics and therapeutics are described below.

**IMPROVED DIAGNOSTIC TOOLS AND STRATEGIES FOR VISCERAL LEISHMANIASIS**

Diagnostic tests for VL need to be both highly sensitive (as failure to treat a case is likely to be fatal) and specific (as the drugs are costly, relatively toxic, and typically involve a long and invasive course of treatment). While parasito-
logical examination of spleen and bone marrow aspirates is the most specific test, it too is a highly invasive procedure requiring specifically trained clinical personnel. The direct agglutination test (DAT) is increasingly used to assist diagnosis and reduces the need for bone marrow aspiration. However, clinically suspected VL patients typically attend rural health posts, clinics or district hospitals where facilities for (and expertise in) standard serological diagnostic testing (including DAT) are not always available; there are also concerns about the variability between batches of antigen. The clear need for an inexpensive, rapid and effective diagnostic test for VL has led to the recent development of immuno-chromatographic tests (dipsticks), currently manufactured by two companies, as well as novel antigen detection tests, notably a latex agglutination test for diagnosing antigen in urine.

A TDR-supported multicentre trial is ongoing in East Africa and the Indian subcontinent, comparing the effectiveness of one of the commercially available dipsticks with freeze-dried DAT and a urine latex agglutination test. The effectiveness of the dipstick differs between the two regions, and concerns over specificity remain – especially in relation to the differential diagnosis between disease and asymptomatic or healed infections. Diagnosis of apparently relapsed VL patients is a particular challenge. Hence, the need for a direct comparison of the two currently available dipsticks remains a priority. In addition, such a multicentre trial should incorporate regions endemic for VL caused by *L. infantum*, where sensitivity and specificity is likely to differ considerably. Improvement of the antigens used in the urine dipstick test should also be explored. As improved rapid diagnostic tests become available in the future, the LCTG should have the capacity to respond without delay and carry out systematic multicentre trials across all regions endemic for VL.

Reliable measurements of specificity, sensitivity and predictive values (as generated by the trials recommended above) are necessary but not sufficient for developing a leishmaniasis test-treatment strategy which optimizes the use of limited resources. This requires the development and validation of diagnostic algorithms for making clinical decisions related to suspected leishmaniasis patients. Such algorithms will depend, self-evidently, on locality-specific clinical features (associated with both parasite and human population and disease heterogeneities), and also on the health system context – on the optimal case management strategies that accord with the resources and facilities available where patients attend. The benefits of this approach are illustrated by the policy in Sudan where, during epidemics, clinics can be overwhelmed by patients requiring immediate attention. In such circumstances, suspected VL patients are first tested by DAT. High-titre patients are given treatment, and low-titre patients are examined for differential diagnosis. Only intermediate-titre patients are examined by bone marrow or spleen aspiration. Analogous diagnostic algorithms should be developed and validated for leishmaniases worldwide. Clinical management decisions should also be optimized by the development and validation of algorithms determining, for example, whether to extend a treatment course or switch to a second-line drug.

Secondary priorities for research aiming to improve *Leishmania* diagnostics include:

- evaluating diagnostic tools for post kala azar dermal leishmaniasis (PKDL)
- comparing currently available rapid tests for diagnosis of canine leishmaniasis
- evaluating tools for the detection of asymptomatic infection.
Leishmaniasis

IMPROVED THERAPIES AND PATIENT MANAGEMENT

Miltefosine, an alkylphosphocholine, is the first oral drug to be registered for VL. Due to its teratogenic potential, this drug cannot be provided to females of childbearing age, unless contraception can be assured. With these exceptions, trials on the Indian subcontinent have confirmed the safety and efficacy of miltefosine for VL patients in that region; and there is great promise that the regulated provision of this drug, preferably as directly observed therapy (DOT), could significantly improve drug adherence rates. It is now a priority to test the impact of miltefosine in systematic multicentre trials in all VL-endemic regions outside the Indian subcontinent.

Paromomycin, an aminoglycoside, is currently in Phase III trials in India and also shows great promise, not least due to its low price and good safety profile. This is one of the few drugs available that could potentially replace Sb and/or be used in multidrug therapy. It is now a priority to test the impact of paromomycin on VL patients worldwide.

The development and spread of resistance to Sb in India has led to widespread concern that the limited supply of second-line drugs needs to be protected and used judiciously. One obvious approach to prevent the appearance or limit the spread of drug resistance (e.g. to miltefosine) is to provide multidrug therapy. The choice of drug combination will depend on pharmacokinetic properties and toxicity issues. The aim is to provide a combination with low toxicity, high effectiveness (for example, due to synergism), low cost, low risk of developing drug resistance (which may be related, for example, to half life) and shorter course of treatment (to increase adherence rates). As soon as this knowledge emanates from in vivo experimental studies, combination therapies should be tested in systematic multicentre trials on the Indian subcontinent.

Secondary priorities for research aiming to improve treatment protocols include:
- evaluating combination treatments for VL outside the Indian subcontinent
- evaluating treatments for PKDL and ML/CL
- evaluating the implications of antiretroviral therapy in the clinical management of HIV co-infected patients.

IMPROVED PREVENTION STRATEGIES

Insecticide-treated materials, in particular insecticide-treated nets (ITNs), provide one of the most effective methods for reducing man-vector contact and transmission of vector-borne diseases. ITNs have replaced residual insecticide spraying of houses as the malaria control strategy of choice in much of sub-Saharan Africa and Asia on the basis of comparative cost, logistic effort, sustainability, and (to some extent) environmental impact. Pyrethroids are currently selected for treating the materials on the basis of safety, environmental considerations, and efficacy. It is important to monitor the development of pyrethroid resistance in vectors where pyrethroid-treated materials are widely used, but fortunately pyrethroid resistance has not yet become an operational concern for the various ITN malaria programmes worldwide.

ITNs will be most effective when the vector bites humans mostly inside houses during the night, when people are in bed. Hence, to determine the likelihood that an ITN programme could significantly reduce leishmaniasis transmission in a given locality, it is essential first to have sufficient evidence from the principal
site about the time of human transmission, for example by carrying out sandfly behavioural studies. Where sandflies bite indoors but are significantly active from dusk onwards, insecticide treatment of curtains may provide effective protection. Where people sleep outdoors, and are at high risk of sandfly bites, protection can be achieved by using ITNs hung outdoors.

Recent ITN trials in Syria and Afghanistan have demonstrated significant protection against CL (L. tropica) transmitted by Phlebotomus sergenti, and it can be assumed that this strategy would be effective throughout the range of P. sergenti (dependent on local compliance). The same trial in Afghanistan also showed that insecticide treated bed sheets provided protection against CL, and there were some cost advantages of this approach. Also, a field trial in Venezuela demonstrated the protective effect of insecticide treated curtains (ITC) on transmission of CL (L. braziliensis) transmitted by Lutzomyia youngi. However, to date there is no other direct evidence that insecticide treated materials reduce leishmaniasis transmission.

The effectiveness of current residual insecticide spraying campaigns against VL on the Indian subcontinent and in Brazil is limited by the inherent difficulties of achieving widespread and regular coverage. No such campaigns are ongoing in East African VL endemic areas (where sandfly vectors are largely exophilic). The provision of ITNs (or ITCs) could, in contrast, lead to a significant and sustainable reduction in VL morbidity. It is therefore a high priority to test the impact of ITNs (indoor or outdoor, as appropriate) or ITCs on VL incidence in large-scale field trials where there is convincing entomological evidence that these interventions are likely to be effective. Where such evidence is insufficient, further entomological studies should be undertaken to inform whether trials are justified.

Secondary priorities for research aiming to validate prevention strategies include:

- comparing the effectiveness of competitor insecticides applied to dogs for control of zoonotic visceral leishmaniasis
- evaluating the efficacy of insecticide treated materials in prevention of CL (except for L. tropica regions, where there is already proof of principle)
- optimization of ITNs (materials, mesh size, coolness specifically for sandflies).

Once a prevention strategy has been validated by field trials, there remain considerable bottlenecks impeding the translation of available tools into more effective leishmaniasis control. Whilst efficacy trials can demonstrate the likely impact of implementing an intervention under specified conditions, further implementation research is required for policy-makers to decide where and when it is cost effective to do so. The first requirement is to have reliable information on the leishmaniasis burden of disease at a local level (where decisions are ultimately made). The second requirement is for sufficient understanding of transmission ecology at the local level to know which intervention (if any) is appropriate. Hence, in order that health delivery systems can rationally allocate resources for leishmaniasis control, it is essential that managers have ready access to reliable local data. In practice, this can be most effectively achieved by developing geographic information systems (GIS) into which surveillance data are regularly and systematically incorporated. Distribution maps of burden of disease and sandfly vectors, at a resolution relevant for disease control decisions (e.g. municipality level), need to be generated by the collation and analysis of available data. Gaps can be filled in by producing risk maps fitted by comparison of known distributions with environmental correlates with known compre-
hensive distributions (such as from satellite images). Such an approach has been developed for a wide range of other infectious diseases, and should become an invaluable management tool for leishmaniasis control programmes. The approach should be tested by pilot studies, probably at sub-national level, in different endemic regions to help develop a framework for best practice. The approach should take advantage of existing risk maps for other diseases. The development of low-technology instruments for epidemiological assessment would be an immediately implementable strategy in many endemic foci and could provide data for risk maps when the infrastructure for GIS comes into place.

The rapid assimilation of surveillance data by health managers, combined with data on changes in climate, land cover or human population movements, could provide a basis for predicting epidemics and so allow rapid responses to potential emergencies (such as in Southern Sudan). This requires the development of leishmaniasis epidemic predictive models as well as protocols for emergency response.

Although the number of Leishmania-HIV co-infections has been drastically reduced in industrial countries since the introduction of highly active anti-retroviral therapy (HAART), the ruralization of HIV transmission and the urbanization of leishmaniasis in many low-income countries, where most HIV infection occurs, demand for alertness and surveillance. In addition, the benefit of HIV treatment does not apply to the 50–60% of co-infected patients for whom diagnosis of Leishmania infection precedes or concurs with the diagnosis of HIV. More importantly, in Sub-Saharan African countries, which account for the vast majority of HIV/AIDS global cases, very few HIV-infected patients have access to HAART.

With the development and spread of antimony resistance on the Indian subcontinent, and the potential for introducing new drugs either singly or in combination to combat this threat, it is vital that a rational policy is devised to minimize the spread of resistance to new drugs. Experience with antibiotics and antimalarials indicates that, where drug adherence is poor and there are high rates of transmission, the rate of spread of drug resistance could (in the absence of such a policy) exceed the rate at which new drugs become available. It is therefore a high priority to carry out analytical studies anchored by empirical data to compare the likely impact of different drug policies on the development and spread of Leishmania drug resistance (analogous to the considerable body of analytical work explaining the factors determining the spread of antibiotic resistance).

As new drugs are introduced in accordance with rationally designed drug policies, it is vital to develop a systematic surveillance system for drug resistance so that drug policy can respond rapidly to changing conditions, and to alert clinical practitioners. Currently, resistance surveillance must depend on reports of clinical outcome following patient treatment, validated by drug sensitivity assays of parasite isolates from a sample of patients. But it would be beneficial to base a surveillance system on readily identifiable molecular markers (as they become available).

Effective implementation of leishmaniasis control programmes, whether limited to diagnosis and treatment or including prevention strategies such as vector control, require an appreciation of the limits set by economic constraints and by the structure of health systems. As a concerted attempt is made to exploit the battery of existing and new tools and information (as they become available) to reduce the global burden of leish-
Leishmaniasis, it will be vital to invest in implementation research to ensure sustainability. This should include, for example, cost-effectiveness analyses of competing leishmaniasis test-treatment strategies and competing prevention strategies. Socioeconomic and behavioural studies are also required to evaluate factors determining drug adherence in order to aid the development of tools for increasing the proportion of patients receiving full treatment courses. The cost of not intervening with an integrated control strategy, or of interrupting such a strategy and facing the consequent resurgence of disease, is also an important consideration.

PARTNERSHIPS AND CAPACITY BUILDING

Capacity building in endemic countries should focus on the newly proposed Leishmaniasis Clinical Trial Group (LCTG), which needs to develop strategic partnerships with the Human African Trypanosomiasis Group and other clinical trial networks, such as the European and Developing Countries Clinical Trials Partnership (EDCTP) and Drugs for Neglected Diseases initiative (DNDi), and with industry, such as veterinary product companies manufacturing diagnostic tests for canine leishmaniasis. Clinical trials require capacity in epidemiological assessment and in communication to different stakeholders. In addition, there is an urgent need to increase capacity in health economics among the leishmaniasis research community.
2. Challenges and opportunities

Leishmaniasis remains a Category 1 TDR disease, signifying its status as emerging and uncontrolled. While the reasons for this are many, the need for new and better tools, such as diagnostics, drugs and vaccines, and gaps in the basic knowledge needed to develop these tools, remain serious barriers to the design and implementation of effective control strategies.

CHALLENGES POSED BY GAPS IN BASIC KNOWLEDGE AND LIMITATIONS OF CURRENT TOOLS

Despite a wealth of information about the mechanisms underlying immunity to disease expression following *Leishmania* infection in experimental animal models, the correlates of immunity in human leishmaniasis remain poorly defined. Understanding the mechanisms of innate or acquired resistance in patients with self-limiting forms of cutaneous leishmaniasis, or in individuals with asymptomatic infections, especially involving visceralizing strains, is crucial to the development of effective vaccines and immunotherapies. Animal models that are used to address these issues should more accurately reflect human disease.

There is limited knowledge concerning the host and parasite factors controlling the pathogenesis of non-healing and reactivating disease forms in humans, especially visceral leishmaniasis, PKDL, DCL, mucosal disease and co-infection with HIV. This information could lead to the design of novel treatments to promote cure or to prevent dissemination or reactivation of latent infections, an emerging challenge in VL-endemic areas that are increasingly engulfed by the HIV epidemic.

Epidemiologic studies have shown familial and ethnic clustering of certain forms of leishmaniasis, indicating the existence of inherited factors in susceptibility in humans. The genes controlling these host response defects have not been identified. Human genetic studies could provide critical information for the discovery of key steps in the pathogenesis of *Leishmania* infections and allow the identification of new targets for chemotherapy and vaccination.

There is a need to develop standardized and effective vaccines for both prevention and treatment of various forms of human and canine leishmaniasis. While a number of antigens have been identified that show promise as potential candidate vaccines, none has so far been evaluated in clinical trials. There is a need to identify additional antigens for VL vaccines, validate the potential of promising antigens, and move forward with selected recombinant protein or DNA-based vaccines into clinical development. The choice of target antigens for further development should be based on a number of criteria, including their expression by amastigotes and their ability to confer cross-protection against different *Leishmania* species, especially *L. donovani*. Existing and new adjuvants, and antigen delivery systems need to be explored to elicit effective immune response of long duration. Given the failure of whole cell killed vaccine plus BCG to protect in clinical trials, the advantages and disadvantages of live vaccination with *L. major* (leishmanization) should be re-examined. The ability of live *L. major* vaccines to confer protection against VL needs to be investigated but care must be taken not to introduce new species of *Leishmania* into areas where they do not currently exist. Meanwhile target populations should be identified, and the potential cost effectiveness of vaccination should be assessed.

The main problems associated with the current treatments for VL are toxicity, cost, non-compliance, and resistance. There is a need to develop
novel oral, non-toxic, short-course combination therapies for visceral leishmaniasis. Combination therapies with new and existing drugs need to be explored in humans and in animal models. The various forms of cutaneous leishmaniasis are commonly treated with SbV in a dose of 20 mg/kg for 20 days, but there are few data on whether this regimen is appropriate for all species. A species-based approach needs to be investigated.

Current diagnostics succeed mainly in confirming the Leishmania genus in active cases of disease. New diagnostic tools are needed that will detect infections in active as well as asymptomatic cases, and that will discriminate Leishmania parasites according to species, drug resistant phenotype, and possible strain-specific clinical associations, especially PKDL, MCL, and asymptomatic visceral infection.

The incidence of the human leishmaniasis is increasing due to the growth and redistribution of populations, HIV co-infection, poor levels of hygiene and nutrition, increasing poverty, plus changes in climate, ecology and vector behaviour. The socioeconomic, environmental, and behavioural risk factors for infection and disease need to be identified, especially in peri-urban and refugee populations.

More knowledge about the impact of inequity of access to health care, and of health sector reform, will be needed for interventions involving use of even the best tools (drugs, vaccines, diagnostics) to succeed.

There is limited knowledge of the identity and behaviour of sandfly vectors, and of the role of animal reservoir hosts in many endemic regions. This information is crucial to the design and implementation of control strategies involving, for example, insecticide impregnated bednets or dog collars.

**RESEARCH OPPORTUNITIES TO CLOSE THE GAPS IN BASIC KNOWLEDGE AND TO DEVELOP NEW TOOLS**

The completion of the L. major genome (and the soon to be completed L. infantum genome) is a landmark that for the first time identifies all genes that encode proteins and processes that may be targets for drug discovery, vaccine candidates, and useful for diagnostics. Comparative genomic studies at both the gene sequence and RNA expression levels, along with comparative proteomic studies, can now be undertaken to identify key differences among stages, strains and species of Leishmania that affect the establishment of infection and the development of different types of disease. Identification of antigens present and, ideally, conserved in amastigotes may permit the selection of antigens for potential use as vaccines. DNA microarrays can be useful to identify more species- and strain-specific markers for diagnostic studies and for identification of drug resistant isolates. The identified sequences in conjunction with polymerase chain reaction (PCR) techniques can be used to detect and discriminate Leishmania species and strains within suspected sandfly vectors, animal reservoirs, and in individuals with latent or asymptomatic infections.

Genome-wide analyses of host gene sequences can be used to identify genes linked to disease susceptibility in human population studies, and host RNA expression profiling can be used to identify host factors associated with immunity or with particular disease outcomes.

A number of recombinant proteins and DNA vaccines have already been shown to protect against leishmaniasis in animal models, and some may be ready for clinical development. In addition, a number of novel adjuvants that may be able to potentiate the appropriate immune
response in humans are available, including Toll-like receptor (TLR) agonists like monophosphoryl lipid A (MPL), oligonucleotides (CpGs), imiquimod, and transcutaneous delivery systems. The only proven vaccine to date against human disease is live *L. major* (leishmanization), given on the arm to prevent severe and multiple lesions. The problems associated with live vaccination may be minimized by engineering strains with reduced pathogenicity, or by administering the live vaccine with certain immune potentiating/modulating adjuvants. The use of live *L. major* challenge in validating vaccine efficacy in human should also be evaluated.

Satellite images can be exploited as a source of environmental data to explain the distribution of reservoirs, vectors, and disease.
3. High priorities

INTERVENTION METHODS

- Evaluate rapid diagnostic tests for visceral leishmaniasis.
- Develop and validate diagnostic algorithms for test-treatment strategies.
- Evaluate new treatments for visceral leishmaniasis: paromomycin, miltefosine (outside the Indian subcontinent), and combination therapies on the Indian subcontinent and subsequently elsewhere.
- Evaluate insecticide-treated nets for visceral leishmaniasis control.

STRATEGIES & POLICIES

- Economic, social, and health systems research to enhance implementation of control strategies and drug adherence.
- Develop decision support systems to target control activities and predict epidemics.
- Analytical studies to develop drug policy and design of surveillance protocol to limit and monitor the spread of drug resistance.

PARTNERSHIPS & CAPACITY BUILDING

- Capacity building of new global network: Leishmaniasis Clinical Trial Group.
- Strengthen input of health economics in leishmaniasis control.
- Develop links with clinical trial and surveillance networks, the Drugs for Neglected Diseases initiative (DNDi), and industry.

BASIC KNOWLEDGE

- Comparative genomics and proteomics to identify molecules with roles in disease for development of new drugs, diagnostics, and vaccines.
- Immune correlates of protection against human leishmaniasis.
- Definition of host and parasite factors involved in pathogenesis.
- Human population genetic studies to determine genes controlling susceptibility to VL and response to drugs.
- Identification of new vaccine candidates for VL, and novel adjuvants and delivery systems to promote effective and long-lived immune response.
- Epidemiologic studies to identify socioeconomic, environmental, and behavioural risk factors for infection and disease.

NEW AND IMPROVED TOOLS

- High-throughput technologies for identification of target molecules and development of novel assays.
- Selection and development of candidate vaccines and adjuvants for evaluation in clinical trials, including live *L. major* as a vaccine against VL.
- Development and evaluation of immunotherapeutics.
- Novel oral, non-toxic, short-course treatments for visceral leishmaniasis and combination therapy in VL involving new and existing drugs to improve efficiency and reduce emergence of drug resistant strains.
- Diagnostics that will discriminate between *Leishmania* in active disease, asymptomatic or latent infections, drug resistant phenotypes, and disease associations.
- Tools to identify sandflies at species level and to identify infected flies.
Annex 1

AGENDA: Scientific Working Group on Leishmaniasis
**Day 1, Monday 2 February 2004**

<table>
<thead>
<tr>
<th>Time</th>
<th>Item</th>
<th>Speaker</th>
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<tbody>
<tr>
<td>08.30–09.00</td>
<td>Welcome address</td>
<td>Dr. Asamoa-Baah, ADG/CDS Dr. R. Ridley, Director a.i. of TDR</td>
</tr>
<tr>
<td>09.00–09.15</td>
<td>TDR disease strategic plans and role of the Scientific Working Groups</td>
<td>Dr. J. Lazdins, TDR</td>
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<tr>
<td>09.00–09.45</td>
<td>Current strategic emphases for leishmaniasis research</td>
<td>Dr. P. Desjeux, TDR</td>
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<tr>
<td>09.45–10.00</td>
<td>Meeting objectives and process</td>
<td>Dr. P. Desjeux, TDR</td>
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**STRATEGIC RESEARCH – Dr A. Oduola, STR**

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<th>Item</th>
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<tbody>
<tr>
<td>10.30–11.00</td>
<td>From genomics to discovery</td>
<td>Drs Zilberstein, K. Stuart and A. Fairlamb</td>
</tr>
<tr>
<td>11.00–11.30</td>
<td>Drug resistance</td>
<td>Dr. S. Croft</td>
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<tr>
<td>11.30–12.00</td>
<td>Host genetics</td>
<td>Dr. A. Dessein</td>
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<tr>
<td>12.00–13.00</td>
<td><strong>Lunch break</strong></td>
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**IMPROVED TOOLS – Dr R. Ridley, PRD**

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<tr>
<th>Time</th>
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<tr>
<td>13.00–13.30</td>
<td>Vaccines</td>
<td>Dr. S. Reed</td>
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<tr>
<td>13.30–14.00</td>
<td>Molecular tools for epidemiology</td>
<td>Dr. I. Guizani</td>
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<tr>
<td>14.00–14.30</td>
<td>Drug trials</td>
<td>Dr. S. Sundar</td>
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<tr>
<td>14.30–15.00</td>
<td>Tools/strategies for canine leishmaniasis</td>
<td>Dr. C. Davies</td>
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<tr>
<td>15.00–15.30</td>
<td>Tools/strategies for insecticide-treated fabrics (Dr D. Elnaiem)</td>
<td>Dr. A. Warburg</td>
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<tr>
<td>15.30–16.00</td>
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**IMPROVED STRATEGIES – Dr H. Remme, IR**

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<tr>
<td>16.00–16.30</td>
<td>Cost-effectiveness of diagnostic and therapeutic strategies</td>
<td>Dr. M. Boelaert</td>
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<tr>
<td>16.30–17.00</td>
<td>Leishmania/HIV co-infection</td>
<td>Dr. J. Alvar</td>
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<tr>
<td>17.00–17.30</td>
<td>(Impact of inequity of access to services)</td>
<td>Dr. N.K. Ganguly</td>
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<tr>
<td>17.30</td>
<td><strong>Reception (main cafeteria)</strong></td>
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**Day 2, Tuesday 3 February 2004**

**PARTNERSHIP & CAPACITY BUILDING – F. Zicker/RCS, A. Oduola/STR & H. Remme/IDE**

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<th>Time</th>
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<tr>
<td>08.30–09.00</td>
<td>(Field diagnostic tests)</td>
<td>Dr. J. Seaman</td>
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<td>09.00–09.30</td>
<td>Risk factors</td>
<td>Dr. A. Llanos Cuentes</td>
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<td>09.30–10.00</td>
<td>Can leishmaniasis still be considered as category I?</td>
<td>Dr. N. Saravia</td>
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<tr>
<td>10.00–10.30</td>
<td>Working Groups I, II (jointly)</td>
<td>Salle M. 105</td>
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<td>Working Groups III, IV &amp; V (jointly)</td>
<td>Salle C</td>
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<tr>
<td>10.30–11.00</td>
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<td>11.00–12.30</td>
<td>Working Groups I, II (jointly)</td>
<td>Salle M. 105</td>
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<td>Working Groups III, IV &amp; V (jointly)</td>
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<td>13.30–18.00</td>
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<td>15.30–16.00</td>
<td><strong>Coffee break (Salle C)</strong></td>
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1 Unable to attend

2 Shift in presentation period between Drs Seaman (section II) and Ganguly (section III)

3 TDR definition of category I disease: epidemiological situation getting worse, and incidence of infection and disease increasing.
Day 3, Wednesday 4 February 2004

<table>
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<tr>
<td>08.30–09.30</td>
<td>Plenary report Working Groups I and II</td>
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<td>Discussion on overall prioritization with functional area coordinators</td>
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<tr>
<td>09.30–10.30</td>
<td>Plenary report Working Groups III, IV and V</td>
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<tr>
<td></td>
<td>Discussion on overall prioritization with functional area coordinators</td>
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<td>10.30–11.00</td>
<td>Coffee break</td>
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<tr>
<td>11.00–12.30</td>
<td>Rapporteurs to prepare draft SWG report</td>
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<td></td>
<td>Draft report distributed for plenary discussion</td>
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<tr>
<td>12.30–13.30</td>
<td>Lunch break</td>
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<tr>
<td>13.30–15.30</td>
<td>Plenary presentation, discussion &amp; amendment of the SWG draft report</td>
<td>Chairman Director, TDR</td>
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<tr>
<td>15.30–16.00</td>
<td>Coffee break</td>
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<tr>
<td>16.00–17.30</td>
<td>Discussion, amendment and concluding remarks</td>
<td>Chairman Director, TDR</td>
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Day 4, Thursday 5 February 2004

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<tr>
<td>08.30–17.30</td>
<td>Finalization of the SWG report (chairman and rapporteurs only)</td>
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Annex 2

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Medical Officer/EURO

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Director/EMRO

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Medical Officer (CRD)/AFRO

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Leishmaniasis Research Coordinator

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Dr Philippe Desjeux, Leishmaniasis
Dr Deborah Kioy, African trypanosomiasis
Dr Axel Kroeger, Dengue

Dr Janis Lazdins, Focal point for disease research coordinators
Dr Philip Onyebuoh, Tuberculosis
Dr Yeya Touré, Malaria
Annex 3

STRATEGIC RESEARCH

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3.3 Host genetics in leishmaniasis .................................................. 34

3.4 Mechanisms of pathogenesis – differences amongst *Leishmania* species

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3.6 Vector–parasite and vector–host interactions in leishmaniasis ................. 42
3.1 FROM GENOME TO DISCOVERY IN LEISHMANIA: RECOMMENDATIONS FOR THE DEVELOPMENT OF DRUGS, VACCINES AND DIAGNOSTICS

Kenneth Stuart,1 Alan Fairlamb2 and Dan Zilberstein3
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2 Division of Biological Chemistry & Molecular Biology, Wellcome Trust Biocentre, University of Dundee, UK
3 Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

INTRODUCTION

The completion of the Leishmania major genome sequence is a landmark that, for the first time, identifies all potential proteins that may be targets for drug development, vaccine candidates, and/or useful for diagnostics. However, it is not known how these pathogens utilize the proteins to establish infection, prevent elimination by the host, and cause disease. Recent technological advances in experimental and in silico analyses create a promising environment for generating the knowledge needed to combat the spectrum of leishmanial diseases. These advances can be exploited for comparative genomics at both the gene sequence and RNA expression levels, for parasite and host cell expression profiling at the RNA (DNA microarrays) and protein (mass spectrometry) levels, and for more comprehensive analyses of host immune responses to infection or experimental immunization including detection of T-cell receptor associated peptide antigens (mass spectrometry) and reactivity to specific antigens by ELISPOT and protein arrays. Our recommendations are below.

FUNDAMENTAL STUDIES

Eluciding aspects of Leishmania gene function, physiology, genetic diversity, and host/pathogen interaction have advanced the ability to develop measures to prevent or treat disease. We suggest:

• Genome-wide studies to identify key genetic differences among strains and species of Leishmania that affect the establishment of infection and/or the development of disease.
• Studies to determine sequence or expression differences that correlate with different types of leishmanial disease (visceral, cutaneous, mucocutaneous, diffuse cutaneous) and post-kala-azar dermal leishmaniasis (PKDL).
• Gene expression profiling at the RNA and/or protein level among strains that differ in disease.
• Studies that generate transgenic Leishmania with altered capabilities for producing pathogenesis.
• Studies of host factors that affect the development of disease.
• Genome-wide analyses that predict metabolic pathways and cellular processes, especially in amastigotes.
• Analysis of pathogenesis in skin lesions, liver, spleen, and mucocutaneous lesions.
• Studies of macrophage expression profiles associated with disease and protection.
• Identification of Leishmania proteins that are predicted to affect host functions.

VACCINES

Substantial evidence exists for the ability to develop a vaccine, although the specific correlates of acquired and induced immunity are yet to be defined. Therefore, we recommend support for vaccine development with an emphasis on a genome-wide approach, and suggest:

• Protein-wide analyses (protein microarrays) to identify protein antigens that are predicted to protect against infection or disease.
• Using the same approach to identify amastigote specific antigens.
• Characterization of immune response specific for natural or induced protection.
• Development of live attenuated (recombinant) parasite vaccines.
• Studies that combine vaccine development with analysis of immuno-pathogenesis.
• Studies of epitopes that may cross react between host and pathogen.
• Assessment of protection in mouse model systems and in parallel human analyses.

Mouse model systems have been very fruitful and should continue to be used in combination with genetic manipulation of the pathogens and in concert with examination of human parallels to the experimental mouse model systems. Such studies will advance our understanding of factors that are critical for the development of disease and can lead to insights that could be used to develop preventative or therapeutic measures or for patient management.

DIAGNOSTICS

Current methods of diagnosis of leishmaniasis involve pathology and serology, using patient sera against specific parasite antigens. However, the emergence of genetic engineering allows molecular diagnosis, which includes amplification of species-
specific parasite sequences using the polymerase chain reaction (PCR). We recommend that TDR supports the application of DNA and protein microarray technologies for diagnosis of leishmaniasis as well as for the study of parasite virulence, development, and host-pathogen interactions, as follows:

• In using DNA microarrays for the identification of better and more species-specific markers, the following directions should be encouraged:
  – Comparative genomics of the various pathogenic Leishmania strains/species. These studies should elucidate all possible species-specific genes. In addition, their expression profiles during infection can be assessed.
  – Gene expression of host genes during infection. This can be done using either DNA microarrays of infected vs. non-infected macrophage cell lines, or using patient tissues. The results of these studies should provide important information on host-parasite interactions as well as on host reaction to parasite virulence. The results will also be useful for improved diagnosis.
• Proteomics is an additional tool to study gene expression. Since regulation of gene expression in Leishmania is post transcriptional, the appearance of specific proteins is critical for the study of pathogenesis, drug development and diagnosis. The following directions should be encouraged:
  – Identification and characterization of Leishmania proteins in all developmental stages, using state of the art methodology. This should include post translational modifications such as phosphorylation and glycosylation.
  – Comparative proteomics of pathogenic species.
  – Comparative analyses of gene expression, e.g. mRNA and protein abundance, throughout the life stages of Leishmania, including developing bioinformatics tools to compare mRNA with protein abundance in order to assess whether it is necessary to use both for diagnostics and drug development.
  – Development of microarrays of Leishmania proteins. This can be done by preparing a Leishmania expression library of the whole genome and expressing all in bacteria followed by printing the proteins/peptides on a solid phase, as has been done recently in yeast. This will be useful for studying phenotypic expression in Leishmania and, more importantly, for diagnosis.
• Development of tools for genome-wide based diagnosis in the field, for genomic and proteomic microarray analyses.

DRUGS

Current drug treatment of leishmaniasis is unsatisfactory due to drug resistance, lack of efficacy, toxicity, route of administration, prolonged treatment schedules, high cost, etc. In particular, the clinical value of frontline pentavalent antimonial drugs (pentostam, glucantime) is being seriously eroded by the emergence of widespread resistance in parts of India and Bangladesh. New drugs are required primarily for treatment of the life-threatening visceral form of leishmaniasis and secondarily for the disfiguring mucocutaneous and cutaneous forms of the disease.

The genome sequence provides a substantial opportunity for identifying novel potential drug targets through bioinformatics and database mining. Comparative genomic studies could identify biochemical targets that are common to Leishmania and trypanosomes, thereby reducing the drug discovery and drug development costs of finding a broad spectrum anti-trypanosomatid drug. Many genes that are unique to Leishmania (or better still, are common with trypanosomes but absent from humans) have unknown functions that may be essential for parasite growth or survival. The essentiality/redundancy of such genes needs to be investigated by gene disruption or gene knockout, and the biological or biochemical function of their products established.

WHO/TDR could play a coordinating role in these post-genomic activities. WHO/TDR could also identify potential drug targets with suitable profiles for drug discovery (i.e. those targets that can be readily produced in large quantities using a robust, cheap and reproducible assay method for high throughput screening).

The Leishmania genome also provides new opportunities to further define the mode of action of existing drugs, with a view to developing novel classes of drugs that act against existing targets or to providing a scientific rationale for combination chemotherapy. Drug resistance is a serious threat to current therapy and a more detailed understanding of resistance mechanisms in clinical isolates may reveal novel strategies to circumvent drug resistance. The development of improved diagnostic methods could be useful in monitoring clinical response to drug therapy and as a predictor of drug sensitivity in clinical isolates.

The following activities are recommended:
• Target identification by database mining and metabolic pathway reconstruction (identify novel or unique/different pathways or enzymes common to trypanosomatids).
• Target validation using reverse genetics (identify essential genes for amastigote growth/survival).
• Target characterization (establish biochemical function, molecular mechanism and structure).
• Assay development and high throughput screening (identify lead compounds).
• Screening of lead compounds against whole cells (chemical validation of target; demonstration of selectivity for parasite vs. host).
• Development of transgenic parasites with reporter systems for use in WHO/TDR screening centres (in vitro and in vivo).
• Comparative studies at the DNA, RNA and protein levels in drug sensitive and drug resistant lines (modes of drug action and drug resistance).
3.2 DRUG RESISTANCE IN LEISHMANIASIS (SUMMARY)

Simon L. Croft  
Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, UK

The efficacy of drugs used for the treatment of visceral and cutaneous leishmaniasis is influenced by many factors. These include host factors and pharmacokinetics as well as both intrinsic variation in the sensitivity of Leishmania species and acquired drug resistance due to selection.

Intrinsic variation in sensitivity (susceptibility) has been described in relation to pentavalent antimonials, paromomycin, miltefosine, azoles and other drugs that have reached clinical trials. For several of these drugs, rank orders of species sensitivity of promastigote and amastigote forms have been described from laboratory studies. This has potential impact on clinical treatment, as demonstrated with respect to antimonials, ketoconazole and miltefosine.

Acquired resistance, due to selection, has been reported from laboratory studies on both clinically used and experimental antileishmanial drugs. However, it is only in the past decade that the clinical importance of resistance has become evident as pentavalent antimonials have become increasingly ineffective for the treatment of visceral leishmaniasis in Bihar State, India. Clinical Leishmania donovani isolates from non-responding patients showed a lower sensitivity to pentavalent antimonials than those from responsive patients. The mechanism(s) of resistance to pentavalent antimonials are a subject of current research and are still not clearly understood. Mechanisms involved in resistance to pentamidine, miltefosine, amphotericin B and azoles have been more clearly defined.

The development of resistance to pentavalent antimonials in a clinical focus of visceral leishmaniasis, and the potential of resistance developing to other drugs, has several implications. It makes the search for new drugs more urgent, it focuses attention on our lack of knowledge about the molecular and biochemical mechanisms of action of antimonial drugs on Leishmania, it places emphasis on strategies and policies for use of antileishmanial drugs in endemic areas, and it brings into focus the need to consider drug combinations in antileishmanial therapy. There is a need to develop systems for monitoring and surveillance of the spread of resistance in anthroponotic foci and species specific diagnostic markers in some zoonotic foci where species of different intrinsic sensitivity co-exist. For antimonials and most drugs, this is problematic due to the absence of suitable markers and the reliance on a culture assay to adequately correlate clinical and in vitro resistance.
3.3 HOST GENETICS IN LEISHMANIASIS

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Infections by Leishmania cause a wide spectrum of disease ranging from the asymptomatic to the severely clinically symptomatic (1). Experimental work and studies in endemic populations show that the variety of manifestations in leishmaniasis can be ascribed in part to parasite virulence/pathogenicity and in part to host factors. Immunological studies in human populations have associated certain clinical manifestations with qualitative or quantitative changes in the host specific immune response to Leishmania. These studies, however, do not in general identify the primary defects that cause the complex immunological phenotypes observed. If the genetic make-up of the host is critical in leishmaniasis, genetic analysis will identify these defects and demonstrate the causal link between immunological phenotype and disease.

The genetics of infection by Leishmania was first studied in mice, and mutations in the Nramp1 gene (2, 3) (Bradley et al. 1979; Vidal et al. 1995) and the H-2 locus (4) were shown to affect multiplication of L. donovani. The possibility that susceptibility to leishmaniasis may be increased by mutations at certain genetic loci is supported by linkage studies in mice infected by Leishmania major; several genetic regions have been linked to susceptibility to infection (5–8).

Epidemiological studies have shown familial and ethnic clustering of leishmaniasis phenotypes consistent with the existence of inherited factors in susceptibility to cutaneous (CL) and visceral leishmaniasis (VL) in humans (9, 10). A few genetic-epidemiology studies have indicated that the distributions of disease phenotypes in certain endemic populations are best explained by the segregation of a few major loci (11–13). However, the genetic models proposed are not always convincing, certainly not as convincing as those described for the control of other parasitic diseases (14). Nevertheless, data from experimental models and endemic populations strongly suggest that human susceptibility to leishmaniasis is influenced by host genetics; this control is complex and involves several loci of equal importance.

Early attempts to identify genes involved in susceptibility to cutaneous leishmaniasis in humans have focused on candidate-gene testing (15). Associations have been reported between some HLA alleles (16–18) and TNF gene polymorphisms (19) and the risk of cutaneous and mucocutaneous leishmaniasis, though all attempts to demonstrate an association between HLA and VL have failed (20–23).

Two recent studies in Sudan have begun to study the genetic control of VL in endemic populations. One study was carried out by our group during an outbreak of L. donovani that caused infection of almost all inhabitants of a village on the Ethiopian-Sudanese border (24). Though >90% of the villagers showed immunological evidence of infection, only 30% developed visceral disease. Certain families were more affected than others, suggesting some familial components involved in disease (25). Familial clustering was not, however, as clear as that reported in populations affected by schistosomes (14). An attempt to demonstrate that this familial component was due to a major genetic effect segregating in populations as a mendelian trait was unsuccessful, indicating that the hypothesis of a single major locus is probably not tenable. However, a linkage study carried out on 120 affected sibling pairs, a strategy that accommodates a multigenic control, and scanning the entire genome of the patients, showed that one locus (Chr. 22q12) and probably two genetic loci (Chr.2q22-q23) were controlling susceptibility to VL in that population (26). Given the size of the study sample, the 22q12 locus makes an important contribution to the control of VL. It is not known yet whether one single gene or several genes at this locus is (are) involved in the control. It is particularly interesting that a strong genetic effect exerted by a single locus was reported in the population infected by three Leishmania species (27) (L. donovani 2/3 of the infections, L. infantum [1/3] and L. archibaldi [1/3]), all of them able to cause visceral disease. This indicates that the genetic control is acting across L. donovani complex strains. This study (28) has also revealed that, as in other populations (29), certain ethnic groups are more affected than others; thus, it would be most interesting to determine whether the frequency of deleterious alleles differs in ethnic groups in a manner that parallels susceptibility to VL.

The other study was carried out by Blackwell, El Hassan and collaborators, also in Eastern Sudan in an area situated 70 km from our study village. In this study, higher susceptibility of certain ethnic groups was also observed (29). Only gene testing was reported. Candidate genes were chosen based on observations made in experimental stud-
ies. Linkage of VL with IL4 (encoding IL-4) and of PKDL with IFNGR1 (encoding a chain of the IFN-gamma receptor) was reported (30). Interestingly, the regions containing IL4 and IFNGR1 showed no linkage with VL in our study, although we have not yet tested linkage with PKDL. This may reflect the fact that association studies are more powerful than linkage studies for detecting genes with minor effects on the studied phenotype. The linkage of PKDL with IFNGR1 is interesting since the IFNGR1 locus has been linked to control of susceptibility to severe schistosomiasis (31) and to atypical disseminated infections by mycobacteria (32).

Both studies in Sudan tested the linkage with the SLC11A1 (formerly NRAMP1) region. We found suggestive linkage with polymorphisms in the NRAMP1 gene promoter (28). This result was confirmed in the other study by transmission disequilibrium test analysis (33). Thus the gene encoding NRAMP1, or a closely linked gene, is linked to VL. Given the large body of literature on the role of NRAMP1 in both mouse and human susceptibility to intracellular pathogens (34, 35), these results are consistent with a role, although minor, of NRAMP1 in susceptibility to VL.

Thus studies in animals and endemic populations suggest that the risk of severe clinical leishmaniasis is markedly increased by allelic variants at certain genetic loci. These are the HLA locus, the SLC11A1 gene, and alleles of cytokine or cytokine receptor genes. Additional work in distinct populations is however required to definitively establish the implication of certain of these genes in susceptibility to leishmaniasis.

In conclusion, genetics could provide critical information for the discovery of key steps in the pathogenesis of Leishmania infections and allow the identification of new targets for chemotherapy and vaccination. Drugs targeted at host genetic defects are less likely than pathogen targeted drugs to generate resistant parasites. Genetic studies will also allow the identification of subjects at high risk of severe disease. Such subjects will benefit from targeted prophylactic measures; they will also be evaluated carefully in drug and vaccine trials. Susceptibility alleles are likely to play the same role in various infectious diseases, including diseases prevalent in rich countries; thus, exploitation of the results of genetics for new therapeutic and prophylactic measures should benefit from support and work in other fields.

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3.4 MECHANISMS OF PATHOGENESIS – DIFFERENCES AMONGST LEISHMANIA SPECIES

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Although members of the genus Leishmania share (by definition) specific molecular and biologic characteristics, one of the hallmarks of this family of pathogenic protozoa is the diversity of tropism/disease resulting from infection. With few notable exceptions, the form (visceral, cutaneous, diffuse cutaneous, mucocutaneous) and severity of disease is a function of the infecting Leishmania species together with host genetics and consequent immune response. We are just beginning to appreciate the complexities of these interactions, yet they have import for the development of a vaccine against leishmaniasis. Given the fact that Leishmania species groups differ significantly at the DNA sequence level (estimated to be approximately 15%), it perhaps should not be surprising that their interactions with the mammalian host differ. However, the literature has been focused upon unifying immunologic mechanisms of control, which would be important for the development of vaccine control measures. Genetic studies utilizing the murine model, though, clearly indicate that the genes controlling innate susceptibility to infection differ amongst the species [4, 7, 20]; these data indicate that different immunologic control mechanisms might regulate infection by distinct Leishmania species, even at identical tissue sites. Further, multiple genes apparently control infection in susceptible murine hosts (acquired immunity phase). Although some of these genes are commonly involved in the control of infection to L. major, L. donovani and L. mexicana (i.e. H-2 and H-11), not all genes appear to contribute equally to the control of infection by all Leishmania species [3, 14, 15].

Infection caused by L. amazonensis appears to be readily distinguished from infection due to L. major in a number of significant immunologic features. Analyses of the contributions and effects of such cytokines as IL-10 and IL-12 suggest distinct patterns of immunologic response in L. mexicana and L. major parasites [1, 5, 8, 12]. For L. amazonensis, the mechanisms involved in pathogenesis appear to singularly involve immune subversion. Initial observations of the immune response in the susceptible murine host indicated that a Th2 response was not, in fact, associated with susceptibility to infection [2]; this suggested that the immune interactions for this cutaneous parasite might in fact be distinct from L. major. Interestingly, in the case of L. amazonensis, most mouse strains examined to date have proven susceptible to infection; there are, however, qualitative degrees of susceptibility. Further, CBA and C57BL/6 mice, which are resistant to L. major infection, are susceptible to L. amazonensis. The differences between these two cutaneous species have become more evident based on recent studies in immunodeficient mice (T, B and cytokine [IL-10/IL-12]) [1, 5, 8, 9, 12, 19].

In the case of L. major, as expected, mice genetically deficient in T cells (or more specifically, CD4+ T cells) display an exacerbated infection in comparison to wild type control mice. The mice genetically deficient in MHC class II expression (and thus, CD4+ T cells) lack the capacity to develop a Th1 (or Th2) response to infection [6, 10]. Without the capacity to develop a Th1 response, these genetically deficient mice are incapable of controlling L. major infection. In contrast, L. amazonensis infection in mice genetically deficient in T cells (athymic nu/nu or RAG-2-/− mice deficient in T and B cells), or more specifically CD4+ T cells (MHC class II deficient mice), fail to develop disease pathology or to allow the in vivo multiplication of parasites [19]. Analyses have demonstrated that this apparent parasite containment was not due to the development of a non-T cell immune response nor due to the metastasis of parasites to alternate (non-cutaneous) anatomic sites. Histochemical and immunohistochemical analyses indicated the presence in the wild type mice (C57BL/6) of infiltrating MAC-1+ macrophages packed with intracellular parasites, as well as the presence of CD4+ and CD8+ T cells and the up-regulation of ICAM-1 at the site of cutaneous infection. These results are consistent with an ongoing Th1-like immune response. Further, these infected wild type mice produced IFN-γ and TNF-α/lymphotoxin (LT) but not IL-4 in response to parasite antigen. In short, the cytokine responses were typical of an ongoing Th1-like response; yet, these mice were susceptible to infection. Obviously, other ongoing immune events could modulate the effectiveness of the Th1 response. An evident immune response or similar Th1-like immune response was not observed in the MHC class II deficient mice. In fact, immunohistochemical analyses revealed that the low numbers of parasites present were found either within non-MAC-1+ cells or were located extracellularly within the tissue matrix. Disease/pathology was able to be reconstituted in RAG-2
deficient mice (deficient in both T and B cells) using either splenic cells or enriched populations of CD4+ T cells; the results indicated that these cells, indeed, were responsible for the pathology observed.

Although IFN-γ producing CD4 T cells appeared to reconstitute disease, it was apparent that splenic cells were more effective [19]. Consequently, it appeared that another cell population could be contributing to the pathogenic response. Further studies indicated, in the case of L. amazonensis and another related L. mexicana complex parasite, L. pifanoi, that B-cell deficient mice appeared to be refractory to infection [10]. Interestingly, similar observations have been made for L. donovani and L. mexicana in B cell deficient mice [13, 17], but not for L. major. B cells are known to function as antigen presenting cells and to produce immunoglobulin. B cell deficient mice, transgenically reconstituted [10] to express membrane (function as antigen-presenting cells [APCs]) and/or secreted IgM, were found to be phenotypically identical to the parental B cell deficient mice. However, reconstitution of the B cell deficient mice with immunoglobulin (IgG) specific for Leishmania membrane components made the mice susceptible to L. amazonensis infection. Further FcRγ-deficient mice also were refractory to infection. Hence, it is the production of antibody (specifically IgG) and the uptake of the parasite through the host Fc receptor that is critical to the development of disease. This result is in contrast to observations of infection with L. donovani, where passive transfer of antibody in B cell deficient mice failed to reconstitute their susceptibility to infection; however, antibody apparently reversed the influx of neutrophils into the liver observed in the B cell deficient mice [17].

The interaction between the T cell and B cell compartments in terms of the mechanisms involved in pathogenesis in murine L. amazonensis infection remains to be explored. Obviously, CD4+ T cells are important in providing “help” for the development of a B cell response and the production of antibody. In addition, macrophage cell uptake of opsonized (antibody coated) organisms is known to enhance oxidative burst and killing; this could presumably result in enhanced parasite antigen presentation, and therefore in an enhanced T cell response. Further work needs to be done to explore these possibilities and to understand the nature of the immune response facilitating infection by L. mexicana complex parasites. The role of IFN-γ in infection may be a double-edged sword, especially in the case of L. mexicana complex organisms. Indeed, in vaccine studies the up-regulation of IFN-γ has been observed to correlate with protection against infection with L. mexicana complex parasites, including L. amazonensis [11, 18]. Although important for the activation of macrophages to kill intracellular pathogens, IFN-γ has also been demonstrated in numerous immunologic systems to be responsible for inflammation and the ability of monocyctic and other cells to reach the site of antigen presentation. In addition, earlier work in vitro suggests that L. amazonensis-infected macrophages may be more resistant to activation with IFN-γ [16] than macrophages similarly infected with L. major. Consequently, the balance between macrophage recruitment and level of activation is critical for control of infection; IFN-γ is critical to both processes and the “balance” point (i.e. level of IFN-γ produced) determines control versus pathogenesis. Recent observations in IFN-γ deficient mice further strengthen this view. IFN-γ deficient mice are comparable to wild type control mice in their susceptibility to L. amazonensis infection; this is in contrast to observations for L. major and L. donovani, where IFN-γ/- mice show a very significant increase in susceptibility to infection. However, IFN-γ is critical for protection in vaccinated mice. Consequently, the balance of activation versus recruitment of “safe” target monocyctic cells in wild type immunocompetent naive mice clearly favours the parasite. The question remains though, will exacerbation (defined as greater parasite burdens than those seen in non-vaccinated mice) be observed as protection wanes in a vaccine model. If IFN-γ at low levels favours monocyctic recruitment, and at higher levels results in macrophage activation and parasite killing, a waning immune response could be problematic. This has implications for vaccine strategies, where the time between immunization and subsequent boosts may be important for the overall efficacy of the vaccine.

CONCLUSIONS

In summary, the critical importance of immunoglobulin (B cells) and T cells in the development of disease in the case of Leishmania mexicana complex parasites appears to distinguish this group from other members of the genus Leishmania. The requirement for an immune response of the host is obviously a reflection of the fact that evolutionarily the parasite has only encountered the immunocompetent mammalian “host” (unlike in the immunologist’s laboratory) and hence has developed and adapted to surviving in response to an ongoing immune onslaught by the host. However, this appears to be an extreme case of immune subversion, as without the host’s immune response the parasite fails to thrive. Consequently, without the host immune response it appears that the transmission of these parasites would be seriously hampered.

In terms of development of a vaccine against New
World cutaneous leishmaniasis caused by members of the *L. mexicana* complex however, we need to further understand the mechanisms of pathogenesis as these may have important implications for vaccine development. Antibody and IFN-γ, when high levels are achieved, may represent mechanisms for parasite destruction; however, as the immune response wanes (with time post-vaccination) these could contribute to enhanced susceptibility of the vaccinated host to infection. Further work is required to examine these possibilities.

Acknowledgements

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3.5 CELLULAR IMMUNE RESPONSES (SUMMARY)

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Human infections with *Leishmania* species induce a spectrum of clinical manifestations ranging from cutaneous lesions which spontaneously heal to visceral leishmaniasis which is fatal unless treated. The clinical outcome depends on the strain of parasite but also, largely, on the specific immune response to *Leishmania* antigens. Murine models of infection have been used to characterize the types of immune response influencing both resistance and susceptibility to infection with *Leishmania*.

THE MURINE MODEL OF INFECTION WITH *LEISHMANIA MAJOR*

Upon experimental infection with *Leishmania major*, distinct features of the spectrum of clinical manifestations seen in patients with cutaneous leishmaniasis can be reproduced in inbred mice of different genetic backgrounds. Mice from the majority of inbred strains (C3H/He, CBA, C57BL/6, 129Sv/Ev) develop locally cutaneous lesions, which spontaneously resolve. These mice do not develop lesions after a second inoculation of *L. major* and belong to the resistant phenotype. Mice from a few strains (BALB/c, DBA/2) develop severe and uncontrolled lesions without becoming immune to reinfec tion and are representative of the susceptible phenotype.

Two functionally distinct CD4⁺ T cell subsets, Th helper (Th1) and Th2, distinguishable by the pattern of cytokines they produce upon stimulation in vitro, were described in the late 80s. Th1 cells are characterized by secretion of IFN-γ and lymphotoxin (LT) that are known to activate host defences against intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13 that favour the development of humoral responses protecting against extracellular pathogens. The murine model of infection with *L. major* provided the first correlation in vivo between 1) the development of protective immunity and an expansion of Th1 CD4⁺ T cells in resistant mice, and 2) the expression of progressive disease and the development of a CD4⁺ Th2 cell response in susceptible mice. It is now generally accepted that genetically determined resistance and susceptibility to infection with *L. major* result from the development of Th1 and Th2 responses, respectively.

This murine model of infection with *L. major* has been used to study the mechanisms underlying genetic differences in Th subset maturation. Many results from several laboratories strongly suggest that IL-4, during the early phase of infection with *L. major*, plays an important role in the development of specific Th2 CD4⁺ T cells. In this context, it has been demonstrated that a specific subpopulation of Vβ4 Va8 CD4⁺ T cells provides the IL-4 necessary for Th2 maturation. Furthermore, a single antigen (LACK) from this complex microorganism drives this early IL-4 response that underlies subsequent Th2 cell maturation resulting in progressive disease. However, recent results have shown that the pre-cursor frequency of these IL-4-producing cells and their expansion during the first days of infection are identical in resistant and susceptible mice, suggesting that IL-4 is not the only signal necessary for Th2 differentiation. Since other Th2 cytokines such as IL-10, IL-13 and TGF-β have been implicated in some conditions (particularly when IL-4 is lacking) in the development of Th2 cells during infection with *L. major*, new aspects accounting for Th2 differentiation and consequently for the susceptibility to infection should be assessed. In this context, it has been recently shown that a minor population of CD4⁺ T cells constitutively expressing CD25, the α chain of the IL-2 receptor, controls the importance of the IL-4 produced by susceptible mice early after infection with *L. major*. Furthermore, in resistant mice, these CD25 regulatory T cells appear to allow parasites to persist and effector memory responses in immune mice to be maintained.

Although the role of IL-12 in Th1 cell development is now well documented, the importance of IFN-γ in Th1 development is still debated. In addition to these two cytokines, other cytokines such as IL-18, IL-23 and IL-27 favour IFN-γ production by CD4⁺ T cells, but their exact role in Th1 differentiation and resistance to infection with *L. major* is still not completely understood.

THE MURINE MODEL OF INFECTION WITH *LEISHMANIA DONOVANI*

In murine models of infection with *L. donovani*, the relationship between Th1/Th2 cell development and resistance/susceptibility to infection is not clear. Effective defences towards visceralizing strains depend strictly upon Th1 cells, and acquired resistance is governed by T-cell and macrophage-activating cytokines including IL-12 and IFN-γ, which play a predominant role. Down-regulation mecha-
nisms including Th2 cell responses have also been demonstrated during experimental infection with *L. donovani*. Although TGF-β, IL-4 and IL-13 may exert limited effects in visceral leishmaniasis, available evidence support a central role for IL-10. Indeed, IL-10−/− mice are resistant to infection with *L. donovani*, and the enhanced resistance is accompanied by increased production of IFN-γ and nitric oxide, a radical that is toxic for the parasites. Thus, IL-10 is a critical component of the immune response that inhibits resistance to infection with *L. donovani*.

**HUMAN INFECTION WITH LEISHMANIA**

In humans, the roles of Th1 and Th2 CD4+ T cells and the cytokines they produce are not yet well understood. The role of IFN-γ in protective immunity has been documented but the role of regulatory Th2 cytokines including IL-4, IL-10, IL-13 in the disease susceptibility is still under debate. Although in mice IL-4 is a potent inducer of Th2 cell development, IL-13 has recently been shown to predominate over IL-4 in T cell functions in humans. Indeed, IL-13 was demonstrated to be the predominant Th2 cytokine in localized cutaneous leishmaniasis (LCL) lesions which renders specific CD4+ T cells unresponsive to IL-12, a mechanism that could possibly maintain Th2 cell development and disease susceptibility. In addition, high levels of IL-10 have been shown to be associated with active visceral and mucosal leishmaniasis. Some IL-10 production has also been demonstrated in human LCL due to *L. braziliensis*, and its downregulation of IFN-γ production has been proposed to explain disease. Furthermore, IL-10 expression was significantly higher in the more slowly healing lesions on patients with LCL caused by *L. major* and was a promoter of disease prolongation in patients infected with *L. mexicana*. In human LCL due to *L. guyanensis*, IL-10 was poorly produced in spite of the association of intralesional IL-10 mRNA expression with unresponsiveness to chemotherapy. Another well known regulatory cytokine with anti-proliferative and antigen-presenting cell deactivating properties, TGF-β, has also been implicated in human leishmaniasis. The expression of TGF-β in the lesions of active and chronic human cutaneous leishmaniasis has been clearly demonstrated, and its potential effect on the development of the disease has been proposed. However, recent data have demonstrated that TGF-β can only poorly inhibit IFN-γ production by peripheral blood mononuclear cells (PBMC) from cutaneous or mucosal leishmaniasis patients stimulated with soluble antigens from *Leishmania*, suggesting that TGF-β is not able to reverse IFN-γ-dependent anti-parasite effector mechanisms. Thus, its exact role in disease susceptibility remains unclear.
3.6 VECTOR–PARASITE AND VECTOR–HOST INTERACTIONS IN LEISHMANIASIS

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Current research involving the vector biology of leishmaniasis is severely limited because few laboratories maintain sandflies for study; fewer still address molecular aspects of vector–parasite or vector–host interactions. Key gaps in our knowledge concerning these interactions include the molecular basis for hindgut vs. midgut development that distinguishes the Viannia and Leishmania subgenera; the role of digestive enzymes in controlling differential survival of Leishmania species in the blood-fed midgut; the importance of parasite and sandfly chitinases in escape from the peritrophic matrix and in transmission by bite; identification of midgut receptors controlling species- and stage-specific binding to midgut epithelial cells; factors that promote anterior migration of promastigotes and their stage differentiation, especially metacyclogenesis; the constituents and importance of the biological plug in transmission by bite; the number of metacyclic promastigotes egested by transmitting flies; the importance of salivary secretions in promoting infections in the mammalian host; and the relevance of pre-exposure to sandfly bites to the epidemiology of leishmanial disease. The following is a brief overview of those studies that have described parasite- or vector-derived molecules that play discernable roles in the development of transmissible infections in the fly; as virtually all of the studies have been cited in a number recent reviews, specific references can be obtained from the list of reviews included at the end of this report.

As far as is known, Lutzomyia and Phlebotomus spp. account for virtually all transmissions of Leishmania to humans. Vector–competent species often display remarkable specificity for the Leishmania species they transmit in nature; but many, if not most, phlebotomine species appear to be inherently refractory to the full development of Leishmania. Specific associations have been reproduced in the laboratory; for example, P. papatasi, fed on either experimental lesions or through a membrane, will support the full growth and development of L. major, but not of any other Leishmania species.

The barriers to complete development that have been identified in refractory flies include the digestive enzymes that are induced by bloodfeeding and can inhibit the early growth of parasites in the bloodmeal; the peritrophic membrane, that can behave as a physical barrier to parasite migration out of the abdominal midgut; and the excretion of midgut contents following bloodmeal digestion that can result in the removal of parasites from the gut. The majority of studies that have followed the development of various Leishmania species within inappropriate vectors have not observed an early inhibition of parasite survival and growth. Instead, the loss of infection is associated with the excretion of the digested bloodmeal, suggesting that the inability of Leishmania strains to persist in an inappropriate sandfly is related mainly to their failure to remain anchored to the gut wall via specific attachment sites. The role for the predominant promastigote surface glycoconjugate, lipophosphoglycan (LPG), in mediating attachment to the midgut epithelium has been supported by a number of findings: 1) purified LPG binds to midguts in vitro; 2) LPG completely inhibits the binding of promastigotes to the gut in vitro attachment assays; and 3) LPG deficient mutants fail to attach to the midgut in vitro or to persist in the sandfly following bloodmeal excretion in vivo. The evidence that the polymorphic structures of the phosphoglycan domains of LPG might control species-specific midgut attachment, and by extension species-specific vector competence, is also strong, based on studies in both P. papatasi and P. sergenti. The ability of P. papatasi to transmit only the species L. major has been attributed to the unique, highly substituted nature of L. major LPG that provides for multiple terminally exposed β-linked galactose residues for binding. The LPGs of Leishmania species, or of L. major mutants that lack side-chain substitutions, or that express side chains not terminating in β-linked galactose residues, fail to bind to P. papatasi midguts in vitro, and the parasites that bear these surface structures fail to persist in P. papatasi following bloodmeal excretion.

These findings suggest that gut-associated lectins or lectin-like molecules, which have been described for sandflies, serve as parasite attachment sites, and furthermore that these receptors can vary between different phlebotomine species. A gene encoding for mammalian-like galectin, a lectin family with affinity for β-galactoside sugars, was found only in the midgut cDNA library of P. papatasi, but was absent from libraries constructed from three other refractory vector species (Kamhawi et al., unpublished). Recombinant P. papatasi midgut galectin (Ppgal) bound specifically to L. major strains bearing β-gal-containing branching sugars on their LPGs, and anti-
bodies against Ppgal inhibited binding of \textit{L. major} LPG to the midgut, confirming its role in parasite attachment. The data indicate that midgut galectin provides the molecular basis for species restricted vector competence, as well as the evolutionary pressure for the LPG structural polymorphisms that are unique to \textit{L. major}.

So long as \textit{Leishmania} can generate and maintain high parasite loads in the midgut during and following bloodmeal digestion and excretion, there is little evidence that the final phase of development in the fly, including anterior migration, accumulation behind the stomodeal valve, and differentiation to infective-stage metacyclic promastigotes, varies according to species or strain. Following passage of the bloodmeal, maturation of the infection involves the release of large numbers of parasites from the midgut, preceded or not by their differentiation to metacyclic promastigotes. While further replication and attachment of some promastigotes occurs within the anterior midgut, metacyclic promastigotes themselves have never been seen in division or attachment. This behaviour might be explained, at least in part, by their loss of intrinsic binding potential, controlled by the developmentally regulated loss of \(\beta\)-gal side chains in favour of side-chains terminating in arabino. The detachment of parasites from the midgut during development might also be explained by saturation of binding sites due to released phosphoglycans. The anterior migration of unattached promastigotes to the thoracic midgut and stomodeal valve has generally been attributed to promastigotes following a sugar concentration gradient, formed as the sugar meal is gradually spilled from the crop into the anterior gut. The accumulation of large numbers of metacyclic promastigotes in the anterior regions of the gut, including their presence in the proboscis, may not in themselves be sufficient conditions for transmission by bite. The prevailing view is that, in addition to the presence of infective stage promastigotes in the anterior gut, efficient transmission involves the formation of a biological plug that impairs the intake of blood. This is thought to promote regurgitation of infective promastigotes from the foregut or behind the stomodeal valve as the fly attempts to dislodge the plug from the feeding apparatus. One element of the plug, aside from the mass of parasites themselves, is a gel-like matrix that is morphologically and immunologically identical to the filamentous LPG-related structure, peptidophosphoglycan (PPG), that is secreted by these parasites in vitro.

The relationship of vector sandflies to the leishmanial diseases they transmit does not end with the deposition of parasites into the skin of the mammalian host. Because infected sandflies also inoculate small amounts of saliva, recent studies have examined how modification of the inoculation site by salivary components can influence the outcome of infection. For several different species of \textit{Leishmania}, the co-injection of parasites with salivary gland homogenates of either \textit{Lu. Longipalpis} or \textit{P. papatasi} produced a substantial increase in lesion size and/or parasite burden compared to controls injected with parasites alone. The molecules present in salivary gland homogenates that are responsible for exacerbation of \textit{Leishmania} infection have not been identified, with one exception: the disease enhancing effects of maxadilan, a powerful vasodilatory peptide found in the salivary glands of \textit{Lu. Longipalpis}, have been attributed to its immunomodulatory properties, including inhibition of T-cell activation and delayed-type hypersensitivity (DTH) response.

To the extent that salivary secretions also contain molecules that are immunogenic, the transmission of \textit{Leishmania} by bite into a host previously sensitized to sandfly saliva, including by an uninfected sandfly, will elicit an immune response at the site of the bite and potentially modify the outcome of infection. The exacerbative effect of saliva on infection, seen when mice were coinoculated with \textit{L. major} and a sonicate of \textit{P. papatasi} salivary glands, was completely abrogated in mice pre-exposed to the salivary sonicate. This protection was reproduced following transmission of \textit{L. major} by the bite of infective \textit{P. papatasi} flies: compared to naive mice, mice pre-exposed to the bites of uninfected flies showed a reduction in lesion pathology and a reduction in parasite load. The protection conferred by pre-exposure of mice to saliva was associated with a strong DTH response as well as up-regulation of INF-\(\gamma\) and IL-12 at the site of bite, suggesting that within this inflammatory setting, infected macrophages might be activated for early killing of the parasites. The induction of a \textit{Leishmania}-specific Th1 response might also be accelerated. The powerful protection against cutaneous leishmaniasis that results from pre-exposure to saliva indicates that the immunogenic salivary molecules might be used as components of an anti-leishmanial vaccine. A DNA vaccine encoding one such protein from \textit{P. papatasi} saliva conferred strong protection in mice against \textit{L. major} plus saliva. Protection against \textit{Leishmania} infections conferred by pre-exposure to sandfly bites might explain why, in areas that are endemic for cutaneous leishmaniasis, the indigenous inhabitants, who are mostly bitten by uninfected flies, generally show attenuated infections compared to newcomers such as tourists or immigrants. The hypothesis that induction of an immune response against com-
ponents in vector saliva can facilitate induction of a protective response against leishmaniasis is supported by field observations in an area endemic for visceral leishmaniasis in Brazil, where individuals who experienced seroconversion against *L. chagasi* antigens did not have an increased anti-saliva antibody response, while those who developed a positive anti-*L. chagasi* DTH response, a correlate of immunity, had increased anti-saliva antibody levels.

**References**


Annex 4

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4.1 VACCINES

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INTRODUCTION

Vaccination against human cutaneous leishmaniasis has been practiced for centuries. Deliberate inoculation of virulent organisms from the pus of an active lesion is an ancient practice. Promastigotes of *Leishmania major* grown in culture were first used in Russia in 1937 by Lawrow and Dubowokoj as a means to effectively induce protection against natural infection. More recently, standardized inoculums of culture promastigotes were developed by Israeli scientists and used in several trials. This process, known as leishmanization, is still used in some countries, notably Uzbekistan. Leishmanization has been shown to be efficacious against Old World cutaneous leishmaniasis. However, several basic and logistic problems have precluded the widespread use of this procedure to prevent cutaneous leishmaniasis, including difficulty in standardizing the virulence of the vaccine and the occasional occurrence of severe and persistent lesions resulting from the inoculum. Vaccination using crude antigen preparation obtained from promastigote forms of various species of *Leishmania* has been tested in clinical trials in humans in both the Old and New Worlds. The results vary from 0–75% efficacy against cutaneous leishmaniasis, with only modest protection afforded against visceral leishmaniasis. Although none of these crude vaccine approaches is ideal, they do offer some support for animal data which indicate that induction of protection against leishmaniasis is feasible and can be achieved with either a viable vaccine or with parasite components.

Many *Leishmania* species infect mice, hamsters, and non-human primates. For vaccine studies, most investigators have used BALB/c mice and *L. major* infection. Laboratory animals are usually challenged by cultured promastigotes without the components of sandflies, which have a profound impact on the fate of the infection. Sometimes amastigotes are injected i.v., as in infection with *L. donovani* (or *L. infantum*) in dogs, mice, and hamsters. Infection using exudates of sandfly salivary glands mixed with cultured promastigotes has been employed in mice and monkeys, and Belkaid et al. have developed a model by which infected sandflies deliver the parasite to the ears of mice.

Because dogs are a natural host for *L. infantum* (*L. chagasi*), they represent a special case. Dogs can be used as models for human visceral leishmaniasis (VL) as well, but are also legitimate vaccine targets in their own right. In southern Europe, pet owners are faced with the problem of incurable VL, and the potential for this to be a significant problem in dogs in the US was recently highlighted. More importantly, dogs play an important role as reservoirs of VL in southern Europe and Latin America, where control of infection in dogs would lead to significant reduction in human infection.

*Leishmania* parasites exist in two principal forms: the promastigote, a flagellated form in the invertebrate host that can be grown in cell-free tissue culture media; and the amastigote, a round intracellular form found in the vertebrate host’s macrophages. There is some differential antigen expression in the two forms, and most vaccine candidates are selected based on their presence at least in the amastigote, though their presence also in the promastigote is an added advantage. Antigens associated with differentiation of the parasite in the insect vector, particularly lipophosphoglycans of the promastigote surface, are considered as possible antigens for transmission-blocking vaccines, as are components of sandfly guts. Specific antibodies to these antigens transferred to the sandfly during the bloodmeal could, in principle, prevent normal maturation of the parasite within the vector.

ANTIGENS FOR THE DEVELOPMENT OF SECOND GENERATION VACCINES

One of the first antigens to be explored extensively in animal models is the promastigote surface protease gp63. The native or recombinant protein and peptides have been used in several vaccine studies with varying degrees of success, perhaps due to differences in expression of gp63 on amastigotes. More recently, LACK, a molecule associated with Th2 responses in BALB/c mice, was shown to have excellent vaccine potential against *L. major*, but not against *L. donovani*. The potential of using LACK as a vaccine to inhibit development of Th2 responses is an intriguing possibility. Other antigens with vaccine potential include the GP anchored PSA-2, P-8, gp46/M-2, cysteine proteases (CP), glucose regulated protein (GRP78), HASPB1 (K26), and the fucose-mannose ligand (FML). All of these antigens have shown potential in animal models of one or more forms of leishmaniasis. A systematic approach to determine which of these is/are most appropriate for clinical development would be useful.

Another intriguing target for vaccine development
against leishmaniasis is sandfly saliva. Recent observations in mouse models point to a protective effect of the phlebotomine’s saliva components against challenge with \textit{L. major}. The mechanism of this protection has not yet been completely elucidated. However, it has been suggested that immunization of mice with the saliva of \textit{P. papatasi} induces a strong delayed type hypersensitivity (DTH) to the saliva components. When parasites are delivered with salivary gland excretions during a bloodmeal, a local delayed type hypersensitivity reaction is induced which could mediate killing of parasites. Inclusion of sandfly components that may induce hypersensitivity in a vaccine given to normal individuals who are exposed to the bites of sandflies (mostly uninfected) may not be without risks.

**ADJUVANTS IN LEISHMANIASIS VACCINE DEVELOPMENT**

Most vaccine studies aim to limit parasite replication in the vertebrate host. Several investigators, including us, have, over the past decade, searched for genes encoding leishmanial proteins that could induce protection against cutaneous and visceral leishmaniasis in experimental models of the diseases. However, identification of candidate antigens is not enough. Appropriate antigen delivery to induce the right type of immune response against leishmaniasis is another critical component of an effective vaccine, i.e., the induction of a strong antigen-specific Th1 response. The two adjuvants approved for human use, alum and squalene, induce potent antibody responses but are poor inducers of antigen-specific Th1 responses. Several different strategies, including IL-12, live vectors, naked DNA, oligonucleotides (CpG sequences), and monophosphoryl lipid A as a stable emulsion (MPL\textsuperscript{®}-SE) have been evaluated. MPL\textsuperscript{®} has been used as an adjuvant in several safety and immunogenicity clinical trials in humans, including in vaccines for malaria, hepatitis B, genital herpes, and allergy desensitization. These studies all found that MPL\textsuperscript{®} was well tolerated and there was no evidence of systemic toxicity. We thus evaluated and compared the immunogenic and protective efficacies of formulating Leish-111f (a promising recombinant vaccine candidate) with either the naturally derived disaccharide adjuvant of \textit{Salmonella minnesota}, monophosphoryl lipid A (MPL\textsuperscript{®}) or with squalene (MPL\textsuperscript{®}-SE).

One of the drawbacks of IL-12 is its inability to stimulate strong immunological memory to the immunizing antigen. Thus, vaccination of BALB/c mice with the leishmanial antigen LACK mixed with IL-12 as adjuvant resulted in short-term protection against challenge with \textit{L. major}. In contrast, vaccination with LACK-DNA induced long-term protection. In addition, these studies suggested that the short-term protection could not be overcome by creating a depot condition after the mice were immunized with LACK plus IL-12. Thus, mice immunized with a mixture of LACK + IL-12 + alum developed a strong Th1 response to LACK but short-term protection could not be overcome by the generation of depot. These results question the utility of IL-12 for general use in vaccine development. Moreover, at the present time, it is difficult and expensive to manufacture IL-12 on a large scale, imposing serious logistics restrictions to its use for mass vaccination in humans.

Although DNA vaccines are still in the relatively early stages of development, experimentally they have been shown to induce excellent protection against several intracellular pathogens including \textit{Leishmania}. Recent studies have demonstrated that DNA encoding the leishmanial proteins LACK, LmSTI1, and TSA could effectively immunize susceptible BALB/c mice against \textit{L. major} by inducing CD4\textsuperscript{+} and cytotoxic lymphocyte (CTL) responses. So DNA immunization may provide an effective way to deliver leishmanial vaccines. However, the DNA vectors currently available are less effective in stimulating appropriate immune responses in humans, even when very large amounts are used, and there is need for significant optimization before this delivery system can be considered for a \textit{Leishmania} vaccine. CpG ODN alone has been shown to induce a state of partial resistance in BALB/c mice for up to five weeks against challenge with \textit{L. major}. If CpG ODN is injected in conjunction with leishmanial soluble antigen (SLA), significant protection is obtained in these animals that is maintained for as long as six months. In these experiments, the immuno-stimulatory properties of the CpG ODN were associated with stimulation of IL-12 and the emergence of strong Th1 response to SLA. Further studies are required to optimize CpG ODN as an adjuvant for humans, largely for safety concerns. Several other adjuvant and delivery systems have emerged during the past decade that deserve attention also. These include vectored (adenovirus, pox virus) DNA, peptides targeting antigen-presenting cells (APCs), water-in-oil emulsions, oil-in-water emulsions, and others. Because this area is one of such active interest, we will focus on adjuvant/delivery systems that may applicable to VL vaccine development, both in terms of efficacy and practicality/affordability.
CLINICAL DEVELOPMENT OF A SECOND GENERATION VACCINE

Based on the protection seen in mice and non-human primates, we selected three leishmanial antigens to be included as a single poly-protein in a recombinant vaccine. The vaccine will be developed both for prophylaxis as well as for therapy of different forms of leishmaniasis. The three antigens are:

- **TSA**: This novel protein of *L. major*, which has sequence homology to eukaryotic thiol-specific-antioxidant (TSA), was discovered in experiments performed to characterize the immune responses elicited by *L. major* promastigote culture filtrate proteins (CFPs). Southern blot hybridization analyses indicated that there are multiple copies of the TSA gene in all species of *Leishmania* analysed (*L. tropica*, *L. donovani*, *L. infantum*, *L. amazonensis*, *L. braziliensis*, *L. guyanensis*). Northern blot analyses indicated that the TSA gene is constitutively expressed in *L. major* promastigotes and amastigotes. Immunization of BALB/c mice with recombinant TSA protein resulted in the development of strong cellular immune responses and conferred protective immune responses against infection with *L. major* when the protein was combined with IL-12.

- **LmSTI1**: Screening of an *L. major* amastigote cDNA library with sera from *L. major* infected BALB/c mice identified one clone with strong homology with eukaryotic stress-inducible protein-1, designated as LmSTI1. LmSTI1 contains six copies of the tetratricopeptide consensus motif that is common to stress-inducible proteins. Recombinant LmSTI1 protein plus IL-12 elicited a mixed cellular response that was skewed towards a Th1 phenotype and protected susceptible BALB/c mice.

- **LeIF**: *Leishmania* elongation and initiation factor (LeIF) was identified by expression cloning using sera from a patient with mucosal leishmaniasis to screen an *L. braziliensis* genomic library. Immunoreactive antigens were purified and analysed, in patient T cell assays, for ability to stimulate proliferative responses and preferential Th1 cytokine production. Several cDNAs were identified, one of which was LeIF. An *L. braziliensis* homologue of the LeIF was selected because this unique molecule has two important properties: 1) LeIF is a powerful stimulator of the innate immune system for the production of IL-12, IL-18, and IFN-γ therefore a Th1 inducer; and 2) LeIF has immuno-therapeutic properties in mice. It appears that IL-12, or the induction of IL-12, together with specific antigen, provides the necessary stimulus that causes the responding T cells to differentiate to Th1 phenotype. IL-12 has been successfully used as a Th1 adjuvant for a variety of antigens in both the murine and non-human primate models of several infectious diseases including leishmaniasis.

We have performed extensive protection studies with these antigens, individually, combined, and as fusion proteins. BALB/c mice were immunized twice (three weeks apart), subcutaneously, with LmSTI1 and TSA mixed with murine recombinant IL-12. Mice were infected with *L. major* and development of disease was monitored for the next three months. Mice immunized with either LmSTI1 or TSA with IL-12 are protected against disease. In contrast, mice immunized with saline or IL-12 alone developed severe lesions, illustrating the need for an antigen and adjuvant formulation in achieving protection in this study. LmSTI1 as a single antigen induced protection, while a combination of LmSTI1 and TSA was also effective. An antigen combination is likely to be a more effective vaccine, consisting as it does of a broad range of different protective epitopes, and thus is less likely to suffer from MHC-related unresponsiveness in outbred human and dog populations.

The efficacy of LmSTI1 and TSA was also tested in *Macaca mulatta* (rhesus monkeys). This model, obviously used to a lesser extent than the mouse, has been accepted as a system that more closely mirrors human immunity for vaccine development. Monkeys immunized with a preparation containing LmSTI1 and TSA, with the recombinant human IL-12 and alum as adjuvant, mounted excellent protection against challenge with 10⁷ metacyclic promastigotes of *L. major*.

A practical vaccine for use in underdeveloped countries should be safe, effective, long lasting, and as inexpensive to produce as possible. A poly-protein vaccine consisting of a single fusion with multiple antigenic epitopes would be less costly to manufacture than a vaccine consisting of several recombinant proteins. For this reason, a poly-protein comprised of the three priority candidate antigens TSA, LmSTI1 and LeIF, fused in tandem, was made, and referred to as Leish-111f. Recombinant Leish-111f with the adjuvant rIL-12 was evaluated in the mouse model of *L. major* and shown to be protective. The most effective combination was Leish-111f formulated with 20 µg of MPL®-SE, where protection was observed for at least 14 weeks. Mice immunized with both 2 µg and 10 µg of Leish-111f plus MPL®-SE were protected against *L. major* challenge for at least 10 weeks, at which point all the mice in the saline and adjuvant control groups had to be euthanized.
Further studies involved testing the protective dose response of rLeish-111f (2 µg versus 10 µg) with 20 µg of MPL®-SE, and delaying challenge with Leishmania parasites for 3 months. Our studies found that, in combination with MPL®-SE, Leish-111f induces a very potent immune response that protects mice against cutaneous leishmaniasis (CL) when challenged 3 or 12 weeks post immunization. In addition to the delayed challenge studies, the combination of rLeish-111f and MPL®-SE has been tested for its ability to protect against another species (L. amazonensis).

Because the antigens comprising Leish 111f are conserved in species causing VL, including L. infantum and L. donovani, we asked whether the same antigen/adjuvant formulation shown to be effective in models of CL would also protect against VL. Both mouse and hamster studies were performed. Overall, we observed partial protection following immunization with Leish-111f formulated with MPL®-SE. In four of eight mice immunized with Leish-111f plus MPL®-SE, partial protection against L. infantum challenge was observed. A 75% reduction in mean parasite burden was measured at day 42 after challenge in the spleens of hamsters receiving the Leish-111f vaccine compared with hamsters receiving saline or MPL®-SE alone. However, the difference in Leishman-Donovan units (LDU) between those receiving the Leish-111f vaccine and the control groups was not statistically significant. In conclusion, it appears that our current vaccine construct is not optimal as a prophylactic vaccine against VL.

We have demonstrated that our vaccine formulation containing the Leish-111f protein with MPL®-SE was able to confer protection against leishmaniasis caused by L. major or L. amazonensis in BALB/c or C57BL/6 mice, respectively. We have shown that MPL®-SE (20 µg/dose) is the most potent of the nine different adjuvant formulations tested in eliciting protection against leishmaniasis in BALB/c mice. Whether the vaccine contained 2 or 10 µg of rLeish-111f, or whether the challenge dose was increased from 2 x 10^5 to 4 x 10^5, was irrelevant with regard to the protective effect. Of particular importance is the finding that susceptible BALB/c mice immunized with the Leish-111f vaccine were protected whether they were challenged 3 weeks or 12 weeks post-vaccination. This is the only example that we know of in which long-term protection in this CL model has been achieved using a defined protein antigen together with an adjuvant suitable for human use. With support from the Gates Foundation, we have produced good manufacturing practice (GMP) grade Leish-111f, filed an IND application to the FDA, and completed a Phase I clinical trial in the US.

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4.2 MOLECULAR TOOLS FOR STUDYING THE EPIDEmiOLOGY OF LEISHMANIASIS

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ECO-EPIDEMIOLOGY OF THE LEISHMANIASES: FEATURES AND FACTS

The leishmaniases constitute a group of widely distributed infectious, parasitic diseases, which can be cutaneous, mucocutaneous or visceral. They are considered to be public health problems because of the severity of the diseases, their prevalence, and/or the burdens they create. Mucocutaneous and some cutaneous lesions are severely debilitating while visceral leishmaniasis is fatal. Although reported in 88 countries worldwide, only 33 countries consider notification of leishmaniasis obligatory. The distribution of transmission foci follows the distribution of humans involved in the transmission (and the sole source for vector infection); zoonotic, when at least one mammalian reservoir host is involved (WHO, 1990; Alvar, 1997).

The leishmaniases are caused by parasites belonging to the genus Leishmania, which has two subgenera: Leishmania and Viannia. The epidemiology of the leishmaniases is extremely diverse and far from being fully elucidated.

So far, at least 20 species of Leishmania are identified as being pathogenic to humans (Rioux et al., 1990). The differentiation of the parasites and their accurate identification is relevant to eco-epidemiology, clinical diagnosis, and management of patients. Indeed, their clinical presentations can be pleomorphic, and one species/complex (e.g. L. braziliensis, L. infantum, L. donovani, L. aethiopica) can be responsible for more than one clinical form. Most species cause cutaneous disease, but some species are responsible for mucocutaneous and visceral disease (WHO, 1990; Alvar, 1997). The number of reports on atypical clinical presentations of the leishmaniases in immunocompetent and immunocompromised patients is increasing (Nigro et al., 2003; Marshall et al., 2000; Rosenthal et al., 2000; Desjeux, 1999; Angaran et al., 1998; Ibrahim et al., 1997; Morsy et al., 1997; Mebrahu et al., 1993; Ghalib et al., 1992; Naik et al., 1978.). Clinical prognosis differs according to the parasite species, and even for the same species according to the clinical form. Treatment is necessary to stop evolution of the disease or to save life, and the mainstay therapy is pentavalent antimony (WHO, 1990). Variable sensitivity of isolates or species to some of the drugs available has been documented (Croft et al., 1997), and treatment of the disease does not provide sterile cure (Mendonca et al., 2004; Dereure et al., 2003; Schubach et al., 1998). For all these reasons, it is important to be able to identify/differentiate Leishmania parasites according to the clinical form they induce or according to their eco-epidemiological features (Guerbouj et al., 2001a; Cupolillo et al., 2003), and also to differentiate a relapse of a latent infection from a re-infection (Saravia et al., 1990; Morales et al., 2002).

Co-existence of Leishmania parasites with other Leishmania species or flagellates (known or unknown) in the same endemic areas, even within the same vector or host, has been documented (Martinez et al., 2002; Noyes et al., 2002; Strelkova et al., 2001; Cortes et al., 1997; Barrios et al., 1994; Mebrahu et al., 1991; Pratlong et al., 1989; Morsy et al., 1988; Evans et al., 1987). Some of the known Leishmania species have been isolated only from animals in endemic areas, and are considered non-pathogenic to humans (e.g. L. turanica, L. gerbilli, L. arabica). The impact of this co-existence on the clinical or eco-epidemiological features of the diseases is not fully understood. Complete elucidation of the transmission cycle requires identifying the same organism in the different hosts (mammals and vectors). All this justifies the need for high-resolution tools.

Molecular tools are needed to identify the parasites because all species share similar morphology at the different stages of their life cycle (WHO, 1990). For a long time, identification has been conditional on in vitro/in vivo isolation of parasites. Success of in vitro isolation depends on transformation of the amastigotes into promastigotes, then on their ability to grow in the medium used; frequently, bacterial or fungal contaminants are a problem (WHO, 1990). In vivo maintenance of Leishmania parasites in appropriate animal models is only documented for some species (Hommel et al., 1995).

Molecular identification of Leishmania parasites has been addressed using different kinds of technique (e.g. isoenzyme typing, monoclonal antibodies, hybridization with DNA probes, PCR assays). DNA techniques offer multiple possibilities: distinction between the different parasite isolates and their
assignment to taxa, detection of *Leishmania* parasites and their concomitant identification, detection of intra-specific diversity, investigation of the parasites within their hosts or vectors, for example. Different taxonomical levels of resolution (isolate, species, complex, genus) have been achieved using the assays developed, some of which are sensitive enough to be considered for diagnosis of disease and infection, and for eco-epidemiological studies (cycles, infection rates, etc.). However, such tools are not available for all known *Leishmania* species, and it is difficult to differentiate among closely related species.

*Leishmania* parasites represent a biologically diverse group of organisms, whose origins and evolution are still under speculation (Momen & Cupolillo, 2000; Cupolillo et al., 2000). The genetics of these organisms is still poorly understood, and the genome can be very plastic (Cruz et al., 1993). *Leishmania* parasites are supposedly diploid; however, ploidy was shown to vary within a karyotype or between isolates (Pages et al., 1989; Bastien et al., 1992). Different studies have confirmed that *Leishmania* have diverged mainly due to mutations without involvement of large inter-chromosomal rearrangements (Beverley et al., 1987; Wincker et al., 1996). There is also evidence that an active mutational mechanism for loss of heterozygosity (Gueiros Filho & Beverley, 1996), and recombination machinery which allows gene replacement (Cruz et al., 1991), exist. Inter-clonal variations within isolates are also documented (Bastien et al., 1990; Macedo et al., 1992). The occurrence of such mechanisms and/or others (to be identified) in natural conditions, and their involvement in the emergence of “new genetic makeups”, are poorly appreciated. A major consequence for epidemiology is that the correlation between the genetic determinants of the parasite and the pathology or any other biologically important phenotype, e.g. drug resistance, is not well established. *Leishmania* parasites show considerable intra-specific variability, and the existence of geographical populations has been illustrated in some instances (*L. braziliensis*, *L. infantum*, *L. donovani*) (Ishikawa et al., 2002; Cupolillo et al., 2003; Guerboj et al., 2001[b]; Mauricio et al., 2001; Schonian et al., 2000). There are few descriptions of population structure in relation to clinical form or eco-epidemiological features (Guerboj et al., 2001[a]; Cupolillo et al., 2003).

Genetic definition of clinically and epidemiologically relevant strains still needs to be established.

*Leishmaniasis* vectors are female sandflies that belong to two genera: *Phlebotomus* and *Lutzomyia*. Transmission of the parasites occurs when they take the bloodmeal necessary for oviposition. To date, more than 30 sandfly species have been incriminated as vectors in transmission of the parasites, but the list is far from closed (WHO, 1990; Alvar, 1997). In many foci, the vectors still need to be identified. Three major aspects hamper such tasks:

- The number of criteria necessary to demonstrate the vector role of a sandfly, which are difficult to fulfill: congruent geographical distribution of the sandfly and the parasite, trophic preferences of the sandfly for humans and the reservoirs, natural infection of the sandfly with the same parasites encountered in humans and the reservoirs, persistence of the infection in the gut after digestion of the bloodmeal, ability of the sandfly to transmit the parasites upon biting.
- The low infection rates, which necessitate catching and dissecting a large number of flies, and the difficulty of isolating the parasites without contamination.
- The difficulty of identifying the sandfly species or of differentiating among closely related organisms (sibling species, subspecies); it is easier to identify the males, but the females are of epidemiological relevance.

Molecular studies are obviously important to the design and development of appropriate tools, to the elucidation of sandfly taxonomy, and to establishing the epidemiological relevance of sandfly spp. In some instances, molecular *Leishmania* typing/detection assays have been used to estimate infection rates, to incriminate vectors, or to address epidemiological issues. Development of sandfly markers and studies of sandfly populations are gaining momentum, but still relate to only a few of the known vectors. Establishment of laboratory colonies of sandflies, when successful, provides an alternative way of addressing their biology and role in transmission, and has also contributed greatly to molecular studies of vectors, which have helped in our understanding of vector–parasite and vector–host interactions. It has principally been shown and is well established that the restriction of some Old World parasite species to their vectors is driven by molecular interactions mediated by parasite lipophosphoglycan and lectin-like receptor structures in the midgut of the sandfly (Sacks et al., 2000, 1994; Kamhawi et al., 2000[a]; Pimenta et al., 1992, 1994), most probably reflecting co-association and/or co-evolution rather than co-speciation (Ready, 2000). The effects of salivary components on the infectivity of the parasites, on modulation of immune responses, and on protection, have been demonstrated and investigated (Titus & Ribeiro, 1988; Belkaid et al., 1998; Donelly et al., 1998; Mbow et al., 1998; Kamhawi et al., 2000[b]; Valenzuela et al., 2001). Host and environmental factors have also been shown to interfere with the
development of Leishmania promastigotes in the vector and with transmission (Warburg & Schlein, 1986; Schlein, 1993; Jacobson & Schlein, 1999; Schlein & Jacobson, 1994[a], 1994[b], 1996, 2001, 2002; Schlein, Jacobson & Muller, 2001). The importance of these different kinds of interaction in the eco-epidemiology of leishmaniasis (e.g. importance of bloodmeal source or plant diet in successful establishment of the intra-vectorial cycle), the impact of vectors on shaping parasite diversity (e.g. selective pressures on the parasites, recombination, genetic exchange, multiple infections), and the impact of each of these interactions on the pattern of leishmaniasis, still need to be fully understood and appreciated.

The reservoir hosts of leishmaniasis are very diverse; they vary according to the parasite species and biotope. The reservoirs of infection include rodents, canids, marsupials, and primates. The concept of ‘reservoir’ involves the ability of this host to maintain the parasite, spatially and temporally, within the transmission area; this infers that the reservoir host is present in sufficient numbers to increase the chances of a sandfly meeting an infected animal. The reservoir host should preferably be gregarious, and live in close relationship with the vector. Furthermore, to ensure the parasite is present perennially in the transmission area, its course of infection should include a chronic component. For human cases to appear, the level of enzooticity should be sufficient and contact between sandflies and humans should be possible. Secondary reservoirs respond only partially to these criteria, indicating an unstable, still evolving or more recent interaction with the parasite. In general, a sylvatic cycle is maintained between the wild reservoir and the sandfly; if either settles in a peridomestic area, this facilitates the development of a domestic transmission cycle, which can include infection of humans. Alternatively, human activities can interfere with the sylvatic cycle and so facilitate the development of a domestic or peridomestic cycle (WHO, 1990; Alvar, 1997). The population biology of the reservoir hosts awaits to be well appreciated, and this knowledge to be translated and integrated into the control of leishmaniasis (Fichet-Calvet et al., 2003). In this regard, it is important to understand: the patterns of infection; how the parasite is maintained over the lifespan and within the populations of the animal host; how migration would contribute to emergence of the disease; the relationship between the reservoir hosts and the vector; the correlation between prevalence and transmission; how the reservoir hosts interfere with the parasite’s biology and diversity.

In fact, each transmission focus should be considered unique because of the diversity of biotopes, hosts, and vectors. On the other hand, changes in epidemiological pattern are registered as a consequence of an increase in risk factors (Desjeux, 2001), atypical clinical manifestations (Nigro et., 2003; Marshall et al., 2000; Rosenthal et al., 2000; Angaran et al., 1998; Ibrahim et al., 1997; Morsy et al., 1997; Mebrahtu et al., 1993; Ghali et al., 1992; Naik et al., 1978.), drug resistance (Lira et al., 1999; Sundar, 2001; Rijal et al., 2003), outbreaks (Marlet et al., 2003; Bhutto et al., 2003; Gontijo et al., 2002; Rowland et al., 1999; Tayeh et al., 1997; Seaman et al., 1996; Jeronimo et al., 1994), HIV co-infection (Wolday et al., 2001; Desjeux & Alvar, 2003), multi-Leishmania infections (Martinez et al., 2002; Strelkova et al., 2001; Cortes et al., 1997; Mebrahtu et al., 1991), or, as recently reported, of mixed Leishmania/Trypanosoma cruzi, or multi-Leishmania/T. cruzi infections (Chiaramonte et al., 1999; Bastrenta et al., 2003). In many instances, knowledge about a specific epidemiological situation in an endemic area/country is obsolete or incomplete, emphasizing the necessity for regular updating using powerful and appropriate molecular methods. The control of leishmaniasis is primarily based on diagnosis and treatment of cases and, whenever possible, on interventions against vectors (insecticide spraying) and/or reservoirs (culling, poisoning) (WHO, 1990). Vaccination constitutes a rational alternative to the control of leishmaniasis. However, in spite of the different generations of vaccines developed, this alternative remains under investigation (Modabber, 2000; Mauel, 2002). The leishmaniases are the result of complex interplay between the environment, the parasite, the host(s) and the vector(s), and the current trend is to consider holistic approaches in improving the control of leishmaniasis. This has allowed new concepts of prediction and prevention of epidemics to be established, based on warning systems involving ecological data (climate, vegetation, population dynamics), monitoring of emerging epidemiological patterns (e.g. emerging foci, atypical clinical manifestations, drug resistance, co-infections), risk factor studies (urbanization, migration, big projects), case mappings, etc.

**MOLECULAR TOOLS AND ECO-EPIDEMIOLOGY OF THE LEISHMANIASES: TOWARDS MOLECULAR ECO-EPIDEMIOLOGY**

Identification of organisms constitutes a key issue in the eco-epidemiology of parasitic diseases like the leishmaniases. Parasitological, clinical or epidemiological features are by themselves insufficient for identification, which has to be based on a molecular approach. However, use of molecular tools is not optimal in disease endemic countries. Current
issues in identification for eco-epidemiological purposes can be classified into three major categories:
1. Establishing a diagnosis in order to identify the etiological agent of the disease, to assess relapse or reinfection, and/or to confirm if a disease is emerging or its clinical pattern is changing. This includes identifying the parasite at the species or variant/strain level, and identifying novel species/strains.
2. Defining/assessing roles within the transmission cycle, which includes: establishing the cycle(s), confirming the epidemiological relevance of a species/subspecies/population/variant (of host, vector and/or parasite), and studies on vector, host or parasite populations.
3. Control and surveillance of endemic or epidemic foci, which includes: eco-epidemiological follow-up of foci, making an inventory of species/populations/variants, monitoring and understanding changes in epidemiological pattern, and predicting or preventing epidemics.

These issues address the parasite, the vector and/or the hosts. Lots of unknowns remain and the new concepts of surveillance are being established. Many tools are still needed. In this respect, recent advances in science and technology are very promising. Molecular tools should no longer be considered as mere supports to eco-epidemiology but as driving tools to monitor and understand the biological complexity of parasites, their hosts and vectors, and ultimately to provide rational ways of controlling and managing the leishmaniases. Besides assigning a species to the infecting organism, incriminating the host(s) or vector(s), or elucidating the taxonomic status of a vector or host, molecular eco-epidemiology would open up the possibilities for establishing working hypotheses to address and understand: the intricate relationships between the parasites infecting a host and between the host and the parasites; the consequences of such interactions on the outcome of the infection; the impact of host/vector interactions on parasite populations and emergence of virulent/pathogenic variants; the importance of environmental change to host and vector populations and to transmission of the disease; the genesis of epidemics, etc.

The concept of using molecular tools in disease endemic countries (DECs) is often still associated with preconceived ideas about cost, conducting experiments in the field, and leaving it to sophisticated laboratories in the North. Molecular tools are needed and are necessary. They call for various levels of expertise. Potential and capacity already exist in DECs; this needs to be strengthened and utilized. All this underscores the need for enhanced capacity building, enhanced resource utilization, promoting mechanisms of scientific interaction among investigators in DECs, training and sharing of resources, complementary expertise and knowledge.

**PROGRESS IN MOLECULAR IDENTIFICATION OF LEISHMANIA PARASITES: A REVIEW**

Over the past 20 years, three major categories of molecule have been considered for use in the identification of *Leishmania* parasites:

1. **Isoenzymes.** Isoenzyme analysis gathers parasites into zymodemes, which are considered as operational taxonomic units (OTU). The experimental protocols used, which are easily adapted, can be considered an advantage. The large-scale use of isoenzyme analysis over a wide range of organisms for a long time has made it a standard technique in genetic studies (Pasteur et al., 1987). Its use in leishmaniases, although limited to certain centres and to different or overlapping sets of isoenzymes, largely supports cycle elucidation (Aljeboori & Evans, 1980[a], [b]; Rioux, 1986; Ben Ismail et al., 1986, 1987; Al Zahraei et al., 1989[a], [b]; Mebrahtu et al., 1992; Bonfonte-Garrido et al., 1992; Campino et al., 1994; Gramiccia et al., 1991; Jimenez et al., 1995; Pratlong et al., 1995; Harrat et al., 1996; Campino et al., 1997) and has allowed description of many parasite species (Peters et al., 1986; Strelkova et al., 1990; Yoshida et al., 1993), and tentative establishment of a classification system for *Leishmania* parasites (Le Blancq, Cibulskis & Peters, 1986; Rioux et al., 1990; Culpilloli, Grimaldi, & Momen, 1994; Thomaz-Soccol et al., 2003). However, with the growing and systematic use of molecular typing, assignment to zymodeme becomes less circumstantial and the biological significance of zymodemes has come under question. A disadvantage of the technique is that it can only be applied to large numbers of isolated promastigotes. It is also time-consuming, and interpretation of the data (numerical analysis, cladistic analysis, population genetics) requires qualified, competent personnel. Because the technique addresses the analysis of proteins, and indirectly the DNA mutations that affect the charge on these proteins, the use of isoenzyme data underestimates the level of genetic variability. The resolution power of electrophoresis has also been questioned.

2. **Monoclonal antibodies.** The development of monoclonal antibodies progressed laboriously and steadily. Although monoclonal antibodies have been acknowledged by several authors as useful for studying parasite taxonomy and evolution (Rioux, 1986; Shaw, 1994; Momen & Culpilloli, 2000), it is
well known that they can target antigens/epitopes which are common to many parasite species or are unique to certain developmental stages. Different species-specific (e.g. *L. tropica*, *L. major*, *L. braziliensis*, *L. naiffi*) monoclonal antibodies have been produced and described as able to differentiate among sets of different *Leishmania* species, but are only used in certain laboratories (Grimaldi & McMahon-Pratt, 1996; Jaffe et al., 1984; de Ibarra, Howard & Snary, 1982; Handman & Hocking, 1982). The formats of the tests developed for *Leishmania* identification correspond to the indirect fluorescent antibody test (IFAT), radio-immunoassay (RIA), or enzyme linked immunosorbent assay (ELISA); they all allow assessment of reactivity to different antibodies in one experiment (Anthony et al., 1987; Shaw et al., 1987; Grimaldi, David & McMahon-Pratt, 1987; Adini et al., 1998). Different kinds of results can be obtained according to the antibodies and test format used, which are scored as: presence vs. absence of signals, proportion of reactive promastigotes, and optical density (OD) units or counts retained. The profiles of reactivity identify the species (by reference to control sets) and gather the parasites into serodemes. Application in epidemiological investigations is largely associated with in vitro or intra-vectorial promastigotes (Grimaldi, David & McMahon-Pratt, 1987; Barral et al., 1991; Bonfonte-Garrido et al., 1992; Barrios et al., 1994; Canto-Lara et al., 1999). A number of limitations have been identified, including; antigenicity is not always well defined; limiting amounts of vector material can interfere with the testing of large batteries of antibodies; false negatives may result from inhibitory factors in the sandfly gut; interpretation of results is very dependent on the antibodies tested and, in particular, a positive or negative reaction with a species-specific antibody does not allow the presence of another parasite species in the sample to be ruled out (Shaw et al., 1987). Also, the possibility that taxonomic specificity might be questioned by extending the use to larger sets of parasite species or genera cannot be ruled out (Shaw, 1994). For these reasons, it is important to always confirm any results obtained by using other molecular assays.

3. DNA targets. The application of DNA probes to the detection and identification of *Leishmania* within biopsies and vectors has been promoted since the early nineteen eighties (Wirth & Pratt, 1982; Rogers, Burnheim & Wirth, 1988). Because they are unique to the kinetoplastida, minicircles of kinetoplast DNA (kDNA) constituted the main target in the initial and many ensuing studies (Arnot & Barker, 1981; Barker, 1987). Restriction profiles of the kDNA minicircles were used to define schizodemes and to compare parasites obtained from humans and other mammalian hosts (Lopes et al., 1984; Cuba Cuba et al., 1985; Pacheco et al., 1986), or for describing parasite species (Yoshida et al., 1993; Peters et al., 1986). Different species-specific probes (*L. major*, *L. aethiopica*, *L. donovani complex*, *L. mexicana*) were developed based principally on minicircle fragments (Lopes & Wirth, 1986; Smith et al., 1989; Laskay et al., 1991[a]; Gramiccia et al., 1992; Ben Hamouda et al., 2000); some of these probes were evaluated for concomitant detection and identification of parasites within touch-blots of dog viscera (e.g. *L. infantum*) (Guizani et al., 2001) or squashes of sandflies (e.g. *L. major*, *L. aethiopica*) (Laskay et al., 1991[b]). It is interesting that, in spite of the limited sensitivity of the minicircle fragment probes in detecting $10^3–10^5$ promastigotes within a dot-blot, the probes are able to detect as few as 50–150 organisms within sandflies or bone marrow, respectively (Laskay et al., 1991[b]; Guizani et al., 2001). It is also interesting to note that the performance of one of the probes, specific to *L. aethiopica*, was shown to be equivalent to the whole kDNA probe and to dissection of sandflies (Laskay et al., 1991[b]). The savings in time are obvious.

Extension of the use of probes to genomic DNA fragments was initially done to confirm the evolutionary relationships between different taxa of *Leishmania*, to estimate the divergence rate, and to infer that *Leishmania* diversity relies principally on mutational mechanisms (Beverley et al., 1987). The scarcity of large inter-chromosomal rearrangements was later confirmed by studies on karyotypes and physical mapping of markers on sets of *Leishmania* species representatives (Wincker et al., 1996). Genomic DNA probes were developed and validated for the identification and discrimination of all Old World species and/or species complexes (Van Eys et al., 1989, 1991; Guizani et al., 1994). The power of such probes is based on the invariant hybridization patterns at the intra-specific level, and on the immediate distinction made between the different species; their potential in detecting hybrids between clearly discriminated species has also been proven (Kelly et al., 1991). Genomic restriction fragment length polymorphism (RFLP) analysis requires lesser amounts of parasites than typing a whole set of isoenzymes, and offers the possibility to de-hybridize the membranes and test other probes when there is an interest in analyzing parasite variability. RFLP markers are co-dominant, so that hybridization profile can be assimilated to genotype and can serve for population genetics and population structure analyses. DNA extraction, generation of restriction digests, transfer of gels, use of radioactive labels, and comparison of hybridization patterns to reference sets for parasite identification are all techniques that require the services of trained personnel; population analyses require competency also.
The polymerase chain reaction (PCR) and its versatile applications entered the world of leishmaniasis in the early nineties (Rogers, Popper & Wirth, 1990). PCR assays aimed at characterizing Leishmania parasites can be of different discriminatory power, differentiating the parasites at species level or isolate level. However, in many instances, only a limited set of species has been referred to while developing the assays.

Besides the classical kinetoplast minicircle targets (Smyth et al., 1992; Lopez et al., 1993; Rodriguez et al., 1994; Nuzum et al., 1995; Bhattacharyya et al., 1996; Belli et al., 1998; Salotra et al., 2001), the PCR targets so far addressed can be divided into five major genomic categories including repetitive sequences (Piarroux et al., 1993; Qiao, Miles & Wilson, 1995; Fu, Perona-Wright & Barker, 1998), arbitrarily defined targets (Mimori et al., 1998; Hanafi et al., 2001) and gene family clusters coding for rRNA (Guevara et al., 1992; Van Eys et al., 1992; Uliana et al., 1994; Cupollilo et al., 1995; El Tai et al., 2000), and miniexons (Fernandes et al., 1994; Harris et al., 1998; Marfurt et al., 2003) or gp63 (Victoir et al., 1998; Mauricio et al., 2001; Guerbouj et al., 2001[b]).

The main purpose of the different PCR assays developed is to identify and differentiate Leishmania species. Some PCR products are unique to only one or a group of parasite species (eg. L. donovani complex, L. braziliensis complex) as a result of amplification of species-specific sequences or use of polymorphism-specific primers (Guevara et al., 1992; Lopez et al., 1993; Qiao et al., 1995; Fu, Perona-Wright & Barker, 1998; Mimori et al., 1998; Hanafi et al., 2001; Salotra et al., 2001). In other cases, distinction between different species rests on the size difference in products of the simple (Smyth et al., 1992; Piarroux et al., 1993; Qiao et al., 1995; Fu, Perona-Wright & Barker, 1998; Mimori et al., 1998; Hanafi et al., 2001; Salotra et al., 2001). In other cases, distinction between different species rests on the size difference in products of the simple (Smyth et al., 1992; Piarroux et al., 1993; Qiao et al., 1995; Fu, Perona-Wright & Barker, 1998; Mimori et al., 1998; Hanafi et al., 2001; Salotra et al., 2001). In other cases, distinction between different species rests on the size difference in products of the simple (Smyth et al., 1992; Piarroux et al., 1993; Qiao et al., 1995; Fu, Perona-Wright & Barker, 1998; Mimori et al., 1998; Hanafi et al., 2001; Salotra et al., 2001).

Potential increase in the sensitivity of detection of infecting organisms constitutes one major advantage of the PCR technique, allowing the difficulties associated with in vitro parasite isolation to be overcome and rendering possible identification of parasites within the patient, host or vector. Only small amounts of DNA or parasite are needed. When tested in defined laboratory conditions, PCR assays can detect less than one parasite. However, the PCR has inherent limitations associated with the detection of low amounts of target DNA against excess background DNA, or with the presence of PCR inhibitors. Therefore, it is important to assess performance of the assay in terms of sensitivity, specificity and predictive value for negative and positive results, as well as in terms of discriminatory power. For instance, if using a species-specific PCR assay in a 'presence vs. absence of amplification' format, it is important to remember that a negative PCR does not necessarily mean the absence of amplification, or that the organism does not belong to the targeted species; also, it does not rule out the presence of other Leishmania species in the sample. It is important therefore to contemplate using other confirmatory tests.

The performance of some of the PCR assays developed has been defined for the diagnosis of Leishmania infections in blood, cutaneous biopsy, or aspirate of bone marrow, spleen or lymph node of human patients (Rodriguez et al., 1994; Costa et al., 1996; Andresen et al., 1996, 1997; Aviles et al., 1999) or dogs (Ashford et al., 1995; Lachaud et al., 2002; Reithinger et al., 2002, 2003). Compared to other diagnostic tests, e.g. direct examination, culture and/or serology, the PCR has proved more sensitive in most cases; specificity is variable, according to DNA target. Use of the PCR for mass screening of Leishmania infections has been questioned (Reithinger et al., 2003).

The application of assays already developed requires
Arbitrarily defined targets have shown potential in the identification of and differentiation between *Leishmania* species (Tibayrenc et al., 1993; Motazedian et al., 1996; Schönian et al., 1996; Noyes, Belli & Maingon, 1996; Guizani, Delligi & Ben Ismail, 2002), and on a few occasions have been considered as phylogenetic markers. However, it has been demonstrated that such phylogenetic interpretation should be cautious and based on molecular characterization of the markers used (Hanafi et al., 2001). Using RAPD for parasite identification constitutes a good alternative because it can be associated with simple interpretation of data; however, it can only be applied to purified or isolated parasites and requires protocols to be well standardized to ensure reproducibility of results (Guizani, Delligi & Ben Ismail, 2002).

Besides identifying the species, or assigning a parasite to a species, DNA techniques can be used to classify the parasite and/or analyse its variability, populations and genetics. The PCR-based assays developed for such purposes refer mainly to targets such as kDNA (Brenerie et al., 1999; Noyes et al., 1998), intergenic sequences of the rRNA gene cluster (Cupolillo et al., 1995; El Tai et al., 2000), gp63 coding or intergenic sequences (Victoir et al., 1998; Mauricio et al., 2001; Guerboj et al., 2001[b]), repetitive sequences e.g. microsatellites (Rossi et al., 1994; Bulle et al., 2002), or arbitrarily defined sequences (Mauricio et al., 1999). The polymorphisms observed have been based on amplification profiles (Brenerie et al., 1999), allelic differences evaluated at the level of the size of the fragments amplified (Rossi et al., 1994), and the sequence of such products (Mauricio et al., 2001; Bulle et al., 2002), their conformation (El Tai et al., 2000) or restriction analysis (Cupolillo et al., 1995; Victoir et al., 1998; Noyes et al., 1998; Guerboj et al., 2001[b]). Genomic RFLP or chromosome size variabilities in relation to copy number of target genes have also been analysed (Victoir et al., 1995, 1998; Inga et al., 1998; Kebede et al., 1999; Guerboj et al., 2001[a]). All these studies have demonstrated intra-specific and even intra-zymodemic variability. In some instances, the genetic heterogeneity of parasites belonging to the same species resulted in a lack of correlation between its well-established zymodeme types and the 'DNA types' identified using various kinds of assay, bringing more fuel to debate about phylogenetic status of such zymodemes (Banuls, Hide & Tibayrenc, 1999; Hide, Banuls & Tibayrenc, 2001). Many of these studies have discussed the polymorphism observed in natural populations of *Leishmania* species but still aim at understanding the genetic diversity of the parasite and the correlation with eco-epidemiological and/or clinical features of the disease. Geographical structuring has been illustrated for various species (Guerboj et al., 2001[a],[b]; Mauricio et al., 1999, 2001; Cupolillo et al., 2003; Schonian et al., 2000). The importance of host/vector-parasite interactions in shaping parasite population structure has been highlighted (Guerboj et al., 2001[b]; Ishikawa et al., 2002; Cupolillo et al., 2003); it has even been demonstrated that the level of *L. braziliensis* parasite variability is correlated with the number of vectors and/or animal reservoirs involved in the transmission cycle (Cupolillo et al., 2003). *Leishmania* genome projects are expected to bring invaluable information and to shed new light on the parasites and their biology (Myler et al., 1999; McDonagh, Myler & Stuart, 2000; Martinez-Calvillo et al., 2003; Saxena et al., 2003; Worthey et al., 2003). Similarly, genome projects will improve knowledge about the parasite’s vertebrate hosts and its hematophagous insect vectors (Tabashnick, 2003; Justice et al., 2003; O’Brien et al., 1999). As a consequence, better design of tools, enhanced appreciation of parasite diversity and biology, and new working hypotheses are anticipated.

It is difficult to recommend one technique over another. Each has advantages and drawbacks. Appropriate assessment of the needs, expectations and limits should help in deciding which assay to select or whether to tailor a new one. In some situations, any of the techniques would appear to be appropriate, while in other cases, the complementary data that each will provide are needed. Development of PCR assays, although powerful, should not prevent us from attempting to improve culture conditions and parasite isolation, since in vitro isolates are still an invaluable resource (Belkaid et al., 1996; Hendricks & Wright, 1979). It is also important to recall that knowledge about the parasite in relation to its transmission cycle has been circumstantial for a long time, and that only in those areas where the team includes a molecular biologist does it tend to become more systematic.
EXAMPLES OF MOLECULAR BIOLOGY APPLICATIONS TO THE STUDY OF VECTORS AND RESERVOIRS

Sandflies constitute a large group of insects, with some 500 species. At least 30 species belonging to the genera Lutzomya or Phlebotomus are considered as proven vectors of leishmaniasis, but the list is far from closed and a lot remains to be known (WHO, 1990; Alvar, 1997). Lutzomya longipalpis is by far the most studied sandfly (Soares & Turco, 2003). Different molecular tools (e.g. monoclonal antibodies, DNA probes, PCR) for detecting Leishmania parasites have been designed, assessed and adapted to meet the criteria necessary for sandfly studies. In other cases, sandfly markers have been developed to address the issues.

Eco-epidemiological application of molecular techniques has included detection of L. aethiopica within naturally infected sandflies in Ethiopia (Laskay et al., 1991[b]), and sequential hybridization of DNA probes specific to P. papatasi and L. major to assess infection rates and their fluctuations in natural sandfly populations in Tunisia (Esseghir et al., 1993). Monoclonal antibodies have demonstrated the co-existence of different parasite species (L. mexicana, L. braziliensis) within the digestive tract of the same vector, Lu. ovum (Barrios et al., 1994), while a Leishmania-specific PCR assay has allowed Lu. verrucarum and Lu. pernuiens to be incriminated in the transmission of leishmaniasis in Peru (Perez et al., 1994). An L. braziliensis-specific PCR-dot blot assay was used to confirm, in an endemic area of Brazil, the hypothesis that infected vectors are clustered, allowing the understanding that the low vector infection rates recorded are indeed the consequence of non-homogeneous distribution of infected sandflies (Miranda et al., 2002).

Taxonomical differentiation of sandflies and vectors has also been addressed using morphometry and molecular tools: isoenzymes, DNA markers (Hodgkinson et al., 2003; Arrivillaga et al., 2002, 2003; Dujardin et al., 1999, 1997; Esseghir et al., 1997). Among the molecular markers showing potential for elucidating the taxonomic status of sandflies are isoenzymes and mitochondrial genes. Isonzymes have proved useful in differentiating between the sandfly vectors encountered in Egypt and in analysing the differences between P. papatasi, P. bergeroti, P. langeponi and P. perniciosus (Kassem Fryauff & Hanafi, 1999). Isonzymes and mitochondrial haplotypes were found particularly efficient at separating genetically divergent populations of Lu. longipalpis and in identifying four different clades (Arrivillaga et al., 2003). Mitochondrial and endosymbiotic markers for P. papatasi were used to appreciate how few were the population differences between peridomestic sites and gerbil burrows in endemic areas in Iran (Parviz, Benlarbi & Ready, 2003). Genes involved in courtship songs and in saliva content have also been studied, and show potential for differentiating among populations of Lu. longipalpis (Oliveira et al., 2001; Bauzer et al., 2002; Yin, Norris & Lanzaro, 2000). The study of these markers is relevant to population isolation, speciation, and mating behaviour of sandflies, or to infection outcome; they could be promising for developing rational ways to manipulate sandfly populations and therefore to control leishmaniasis.

Similarly, application of molecular techniques to the study of reservoirs, and to their incrimination, has been well documented. Principally, isoenzymes have been used to type the infecting Leishmania parasites. Schizodeme analysis and PCR tools have been used to incriminate some Leishmania reservoirs (Lopes et al., 1984; Cuba Cuba et al., 1985; Pacheco et al., 1986; Llanos-Cuentas et al., 1999; Rodriguez et al., 2002; Reithinger et al., 2003). Infection in the dog and the dog’s role in transmission of Leishmania are by far the most documented; fewer studies relate to rodents or other reservoir types. Leishmania PCR assays have been used to assess the presence of L. chagasi in the rodent Proechimys canicollis (Travi et al., 1998), or to follow experimental infections in the spiny rat (Proechimys semispinosus) as an animal model (Travi et al., 2002). Detection of Leishmania using the PCR technique has been applied to proposing a role for the Egyptian mongoose (Herpestes ichneumon) as a reservoir of VL in eastern Sudan (Elaim et al., 2001), and to appreciating the infection patterns in the reservoir host in a study of the ecology of cutaneous leishmaniasis in an endemic area of Israel (Wasserberg et al., 2002). Similarly, the prevalence and seasonal transmission of L. mexicana in populations of woodrats, Neotoma micropus, in an endemic area of Texas were evaluated using a Leishmania-specific PCR assay (Kerr, McHugh & Dronen, 1995); this assay was also used to study the temporal and spatial distribution of L. mexicana infections in a population of Neotoma micropus (Raymond et al., 2003). Finally, morphometry, isoenzyme typing and DNA haplotyping were used to study the geographical distribution and analyse populations of the rodents Psammomys and Meriones in Tunisia (Ben Abderrazak et al., personal communication).

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4.3 DRUG TRIALS

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India

PENTAVALENT ANTIMONY COMPOUNDS

On the Indian subcontinent, pentavalent antimonials (Sb) are fast losing ground as antileishmanial agents; branded products and generic products are similar in safety and efficacy. Drugs from reputed manufacturers should only be used as bad lots can cause serious and fatal toxicity; even with standard products, serious and fatal antimony toxicity is not infrequent (3–6% deaths).

Drug resistance

Resistance to antimony is a serious problem in most parts of the State of Bihar, India, and is slowly spreading to neighbouring areas of Uttar Pradesh and to the Terai regions of Nepal. There is evidence that resistant strains of Leishmania are emerging in Bihar, and a strategy is needed to combat and prevent resistance developing to other antileishmanial drugs. This should include:

- molecular probes/in vitro drug sensitivity assays
- simple, cheap and easily applicable probes
- multidrug therapy.

In other parts of the world, pentavalent antimonials remain the most important antileishmanial compounds for all forms of leishmaniasis, but:

- in both immunocompetent and HIV co-infected patients, chemical pancreatitis has been reported, as has cardiotoxicity;
- the availability of branded products has become difficult; there are not many manufacturers of safe antimony products.

Is there a need to replace antimony with safer antileishmanial drugs for treatment of visceral leishmaniasis?

In view of the serious and fatal toxicity and declining efficacy there is a need to replace Sb with safer and more efficacious compounds.

Pentavalent antimony in cutaneous leishmaniasis

Cutaneous Old World (L. major, L. tropica, L. infantum) and New World (L. mexicana and L. braziliensis complexes) leishmaniasis is commonly treated with Sb at a dose of 20 mg/kg for 20 days, but there are few data on whether this regimen is appropriate for all species. A species-based approach to treatment has been advocated, especially in countries where several species may cause cutaneous leishmaniasis (CL). Intra-lesional Sb has been used with encouraging results in Old World self-healing CL.

PENTAMIDINE

This is the first drug used in refractory patients. However:

- cure rates have declined from an initial ~100% to 70% in recent years;
- it has serious toxicity, e.g. insulin dependent diabetes mellitus or occasional death, and this is unacceptable;
- limited experience in Sudan suggests that pentamidine may not be very effective in refractory cases.

Use of the drug has now been abandoned in India, although short courses are effective, without adverse events, in several forms of cutaneous disease.

AMPHOTERICIN B

In India, this is the most extensively used drug in Sb refractory patients; its efficacy is nearly 100%, with no resistance noted, though occasional relapses do occur. However:

- prolonged hospitalization for infusion of the drug is a severe limiting factor;
- it is a toxic drug and infusion reactions are common; serious toxicity e.g. hypokalemia, nephrotoxicity, myocarditis and death occur not infrequently;
- cardiac and electrolyte monitoring are needed, and the patient has to be kept in hospital;
- most endemic countries do not have the capacity (hospital beds and qualified personnel) to cater for the needs of visceral leishmaniasis (VL) patients;
- the cost of treatment is several times more than with generic Sb.

The drug appears to be effective in other forms of leishmaniasis besides VL, although experience is limited in this respect.
Lipid formulations of amphotericin B

There has been remarkable improvement in the tolerability of these drugs, and there is no organ toxicity. AmBisome has the best safety profile. Short courses are possible with good effect.

However, the cost of the drugs is prohibitive and they are not affordable for most patients. Cheap indigenous lipid preparations are needed, and the prices of existing preparations, especially AmBisome, need to be brought down.

These lipid preparations have not been tested in a controlled manner in the case of cutaneous disease.

Three preparations are available:

Liposomal amphotericin B

(AmBisome; Gilead Sciences, Foster city, CA, USA)

For immunosuppressed patients, AmBisome in a total dose of 40 mg/kg spread over 38 days is recommended, but has not been formally compared with shorter regimens. All co-infected patients relapse.

In immunocompetent patients in Europe and South America, a total dose of 18–24 mg/kg, and in Kenya a total dose of 14–18 mg/kg, given over 10 days, cured 90%-100% of patients.

In Indian VL, 6mg/kg (2mg/kg x 3) cured 100% patients, and 3.75 mg/kg cured 89% patients. In a subsequent study employing a single dose of 5 mg/kg of AmBisome, 91% patients were cured with minimal adverse events. Effective single-dose treatment makes it possible to treat a large number of patients in a very short time. In India, the cost of a single dose of 5 mg/kg of AmBisome for a 30 kg patient is US$ 390, compared with ~US$ 60 for a typical treatment regimen of conventional amphotericin B.

Amphotericin B lipid complex

(Abelect [ABLC]; Intermune, USA)

Similarly, a total dose of 10–15 mg/kg ABLC delivered over 5–10 days cured 90%-100% of patients.

Amphotericin B colloidal dispersion

(Amphocil [ABCD]; Sequus Phrmaceutical; Menlo Park, USA).

In Brazil, five and seven doses of Amphocil at 2 mg/kg cured 90% and 100% of patients respectively, but side effects were a limiting factor.

PAROMOMYCN

Paromomycin, an aminoglycoside, is well tolerated and effective for VL, but less so for CL. The drug has the potential to replace Sb as the primary drug for all forms of leishmaniasis. The safety profile seems to be excellent, and the drug could be used for combination chemotherapy.

At the moment there is no industrial production, but an Indian company has started trial production; the cost is likely to be affordable (in the range of US$ 20–30).

In India, a dose of 16 mg/kg/day for 21 days cured 93% of VL patients. A Phase III trial has already started in India; results will be available by the end of 2004.

Results of parenteral use of paromomycin for treatment of CL have so far been unsatisfactory. In Belize, cure rates of L. braziliensis sores were around 50%, and were lower than this in Colombia. In Brazil, 16mg/kg intramuscular daily for 20 days cured only 33% patients with mucocutaneous leishmaniasis.

Three preparations of paromomycin ointment have been used for CL:

- paromomycin 15% plus methylbenzethonium chloride 12%. In placebo controlled trials in Israel and Ecuador, this produced cure rates of 74% vs. 26% and 72% vs. 9%, respectively, at 50 days. In a double blind, randomized placebo controlled trial in Guatemala, 86% were cured with the drug vs. 39% with placebo.
- 15% paromomycin with 10% urea, a less toxic formulation, had no effect on CL in Iran or Tunisia.
- WR279396, containing 15% paromomycin plus 0.5% gentamicin, accelerated cure time in CL due to L. panamensis, but the cure rate was no higher than with placebo.

MILTEFOSINE

Miltefosine is an alkyllysophospholipid originally developed as an anti-cancer agent; it was found to be leishmanicidal in the late 1980s. Several trials of miltefosine in VL have been carried out in India, where the drug is now approved for the treatment of VL in adults at a dose of 100 mg (~2.5 mg/kg) daily for four weeks.

The drug has been shown to be safe and well tolerated. It has consistently cured ~95% patients, which is comparable with the more toxic reference drug amphotericin. The principal adverse events are mild
(in the majority of patients) to moderate gastrointestinal symptoms, notably vomiting (40%) and diarrhoea, reversible asymptomatic transient elevation of hepatic enzymes, and nephrotoxicity at high doses.

Tolerability and efficacy in children are similar to those seen in adults. Phase IV trials in adults and children have just been completed in India to see whether the promise holds good in the field.

As miltefosine causes abortions and congenital defects in animals, it is not used in women of childbearing age unless contraception can be secured.

Results from trials in CL in Colombia and Guatemala suggest the drug has variable efficacy in this type of leishmaniasis.

It has been suggested that directly observed therapy (DOT) would improve compliance with the drug.

Market prices in India are very high and the drug is not affordable to most of those who need it.

**SITAMAQUINE**

The development of sitamaquine (GlaxoSmithKline), a primaquine analogue whose activity against *L. donovani* was demonstrated in the 1970s, has been slow. In 1994, a dose ranging study in Kenyan VL showed moderate activity. In 2002, a Brazilian study reported a surprising lack of linear correlation between dosage and pharmacokinetics and cure rates.

Methaemoglobinemia is a common side effect, and some patients have developed nephropathy. The drug appears to have a good efficacy in Indian VL; however, there are some safety concerns.

**MULTIDRUG THERAPY**

Experience in India suggests that the few antileishmanial drugs available need to be protected, and used judiciously and discretely.

To improve compliance, the duration of treatment needs to be shortened. The cost of therapy needs to be brought down.

A few combinations, involving two parenteral drugs, are being tried out in Sudan and Brazil. After in vitro, animal safety and toxicity studies have demonstrated synergism, clinical trials, if well controlled, could pave the way for multidrug therapy of VL.
4.4 CONTROL OF ZOONOTIC VISCERAL LEISHMANIASIS

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INTRODUCTION

Control strategies against zoonotic visceral leishmaniasis (ZVL) tend to rely on early diagnosis and treatment of patients with expensive and potentially toxic pentavalent antimonial drugs. In some endemic countries, control programmes are designed to prevent infections in humans by vector control (house spraying with residual insecticides) and reservoir control (culling of infected dogs). Infected dogs are identified either by their clinical symptoms (such as hair loss, weight loss, extended nails) or by serological diagnosis. Although this practice appears to have been effective in China, it is unclear whether it has worked elsewhere. In Brazil, for example, ZVL has increased steadily during the last 10–20 years despite the spraying of 200 000 houses and killing of 20 000 dogs per year. Following annual surveys in endemic regions, dogs are culled if their blood samples are diagnosed positive by immunofluorescence. Although experimental trials indicate that dog control may reduce Leishmania incidence in both dogs and children, concerns over the delays between sampling, diagnosis and culling, and a failure to reduce the number of notified cases, have led to scepticism of the effectiveness of the Brazilian control programme. More effective diagnostic tools may allow culling without delay, but a recent trial of the rK39 dipstick test showed poor specificity (<75%) for diagnosing infected dogs. Given the seriousness of the disease, the unwillingness of dog owners to permit their dogs to be culled, and the inconsistent results of trials designed to test the effectiveness of culling, the need to identify alternative sustainable strategies to reduce the burden of ZVL is clear.

Dipping dogs in insecticide or applying topical insecticide lotions can significantly reduce sandfly bites on dogs and so protect them from infection. The epidemiological impact of topical insecticide treatments on dogs depends not only on the reduction in number of sandflies feeding on them (i.e. the anti-feeding effect) but also on the reduction in survival of those flies that do feed so that they are less likely to transmit Leishmania. But this strategy requires regular re-treatment as the insecticidal effect is short lived. A novel method – use of deltamethrin-treated dog collars – for topical application of insecticide on dogs has recently been developed, which enables the insecticidal effect to persist for up to 8 months. Experimental trials have consistently demonstrated that deltamethrin-treated collars reduce the proportion of sandflies that bloodfeed and survive by up to 90%. In experimental trials, widespread implementation of these collars to domestic dogs in Italy and Brazil reduced their risk of being infected with *L. infantum* by 46–58% (in the first year of the intervention). Collared dogs clearly receive personal protection from canine leishmaniasis, but is there a sufficient “mass effect” to cut transmission to children living in villages whose dogs have been collared (i.e. by reducing the entomological inoculation rate)?

DELTAMETHRIN-TREATED DOG COLLARS

This paper reviews the evidence that widespread provision of deltamethrin collars for domestic dogs could significantly reduce the burden of zoonotic visceral leishmaniasis. Particular attention is paid to the results of a recent matched cluster randomized trial in North-West Iran (funded by the World Health Organization Regional Office for the Eastern Mediterranean), which not only demonstrated an effect on *L. infantum* incidence rates in collared dogs, but also in the children from those villages where dogs were collared.

Eighteen villages were ordered into pairs matched by pre-intervention child prevalence of *L. infantum* infection. Within pairs, villages were assigned randomly as control or intervention. All domestic dogs in intervention villages were provided with collars throughout two transmission seasons. Children and dogs were surveyed on three consecutive occasions at yearly intervals, i.e. pre-intervention in 2000, 1 year post-intervention in 2001, and 2 years post-intervention in 2002. Incidence of *L. infantum* infection was measured by seroconversion and leishmanin skin test (LST) conversion in children, and by seroconversion in dogs.

During the two-year trial, a total of 67 direct agglutination test (DAT) conversions were detected amongst the 1622 follow-up tests in initially DAT negative dogs. After adjusting for age, and pre-intervention village seroprevalence, we found a significant effect of collars in both years: in year 1, odds ratio = 0.45 (95% CI 0.30–0.68), p < 0.001; and in year 2, odds ratio = 0.29 (95% CI 0.13–0.65). Although there was some suggestion that protection increased in year 2 (from 54% to 71%), we found no significant...
interaction between treatment and year (p = 0.187). But incidence did vary significantly with year (p < 0.001), and so we controlled for year in the final model. This final logistic regression model (clustered on village) detected a significant effect of treatment on the risk of DAT conversion per year: odds ratio for DAT conversion in treated villages as compared with control villages = 0.39 (95% CI 0.24–0.63), p < 0.001. Hence, on average, the odds of DAT conversion in dogs from intervention villages each year was reduced by 61%.

During the two-year trial, a total of 115 DAT conversions were detected amongst the 4324 follow-up tests in initially DAT negative children. After adjusting for age, LST status and pre-intervention village seroprevalence, we found a significant effect of collars in both years: in year 1, odds ratio = 0.56 (95% CI 0.36–0.89), p = 0.013; and in year 2, odds ratio = 0.47 (95% CI 0.26–0.87). Although, as for seroconversion rates in dogs, there was some suggestion that protection increased from 44% to 53% in year 2, we found no significant interaction between treatment and year (p = 0.518). But incidence did vary significantly with year (p < 0.001), and so we controlled for year in the final model. This final logistic regression model (clustered on village) detected a significant effect of treatment on the risk of DAT conversion per year: odds ratio for DAT conversion in treated villages as compared with control villages = 0.47 (95% CI 0.33–0.67), p < 0.001. Hence, on average, the odds of DAT conversion in children from intervention villages each year was reduced by 53%. The entomological explanation for these epidemiological effects was confirmed by experimental field trials testing the impact of collars on both bloodfeeding and mortality rates of local sandfly vectors.

Following a pilot study using a series of focus group discussions, a questionnaire was designed and given out by local health workers to 1872 householders from a total of 50 villages throughout the endemic region. The answers to the questionnaires provided a measure of the local’s knowledge and attitude to visceral leishmaniasis, the function of domestic dogs in the region, the effort and expense that dog owners provide for their dogs (e.g. in relation to health), and the attitude of dog owners to the collars provided in the trial.

The overall conclusion is that the collars appear to be popular with dog owners, and they clearly reduce the risk of *L. infantum* infection in dogs and hence transmission to children. Collar loss rates averaged 5% during each transmission season, and the turnover rate of the dog population was about 13% per year. These rates are reasonably low, and should not provide a great logistic challenge for health workers to maintain high dog collar coverage rates during a transmission season in Iran (which only lasts 2–3 months). However, it appears that transmission to children was reduced by less than the protection received by collared dogs, and even two years of collaring dogs failed to completely eliminate transmission in the villages. This could be because (1) transmission continues in sylvatic reservoirs which visit villages frequently (e.g. foxes and jackals); (2) the culling rate of stray dogs is inadequate; or (3) some transmission takes place outside villages.

**CONCLUSION**

The duration of the sandfly transmission season, the dog population turnover rate, and the rate of collar loss will all impact on the logistics of adopting this approach elsewhere, e.g. in Brazil. Nevertheless, we remain hopeful that community-wide use of treated collars could provide an effective alternative to dog culling. Prior to making recommendations that insecticide treated collars should be introduced as a control measure in Iran or elsewhere, it is paramount that their impact on disease (visceral leishmaniasis in children) is measured, in order to judge the cost-effectiveness of this intervention. The trials described in this presentation only measured the impact on infection. In order to detect an effect on disease, a much larger study population is required. With the support of WHO (the TDR Director’s Initiative Fund), such a trial is currently ongoing.
4.5 POST KALA-AZAR DERMAL LEISHMANIASIS

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INTRODUCTION

Post kala-azar dermal leishmaniasis (PKDL) is a dermatological form of leishmaniasis that develops in visceral leishmaniasis (VL) patients during or after treatment with pentavalent antimony. PKDL prevails in VL endemic areas, and has been reported in India, Nepal, Bangladesh, Sudan, Ethiopia, Kenya, the Mediterranean Basin, and sporadically in South America. The incidence of PKDL varies from 5–10% in treated VL patients in India to 30–60% in treated patients in Sudan. A few patients with no history of VL develop PKDL. PKDL lesions develop 1–13 months post antimony treatment in Sudan, and 1–3 years post antimony treatment in India. The lesions are confined to the skin and range from macular-maculopapular, to papular, and nodular; they usually start around the mouth then extend to the face (grade 1). Lesions can extend from the face to the chest, back, upper arms and legs (grade 2) and can become generalized (grade 111); they affect areas of the body directly exposed to sunlight.

The age group of 4–8 years is more affected by PKDL in eastern Sudan, with younger children developing more severe lesions while, in India, young adults are more affected. PKDL age ranges are similar to those of VL.

THE PARASITE

It was previously thought that PKDL was caused by specific Leishmania parasite isolates. However, later reports showed that parasite isolates from PKDL patients belonged to different zymodemes (L. donovani MON 18; L. infantum MON 30, MON 267; L. archibaldi MON 82) which were also isolated from VL patients. Furthermore, characterization of seven paired isolates from the same VL/PKDL patients showed they were identical in their isoenzyme profile and gp63 polymerase chain reaction (PCR) restriction profile (RFLP), suggesting that PKDL is caused by the same parasite isolate that causes VL. Typing of unpaired PKDL parasite isolates collected from the same VL endemic area in eastern Sudan showed that the majority of the isolates (13/15) belonged to L. archibaldi based on their isoenzyme profile (MON 82) and gp63 PCR RFLP. The remaining two PKDL isolates were typed as L. infantum (MON 30). Interestingly, in a separate study conducted in the same endemic area, four out of six (4/6) PKDL isolates were identified as L. donovani (MON18) and two as L. infantum (MON 30, MON 267). The ratio of the different Leishmania species in PKDL isolates was similar to their ratio in VL.

PATHOGENESIS

Development of PKDL seems to be a complex mechanism that involves several host and parasite factors. Osman et al. (1998) showed that the presence of PCR detectable parasite DNA (persistence of infection) in VL patients after the completion of a full course of pentostam treatment increased the risk for developing PKDL. Our recent study on typing paired VL/PKDL isolates from eastern Sudan showed that the parasite that causes VL is also capable of inducing PKDL, a finding which suggested the tendency of these parasites to cause skin pathology possibly through differential gene expression. The host immune response in VL patients (TH2) is characterized by increased secretion of IL-10, identified as an indicator for increased risk for developing PKDL.

It is known that following the treatment of VL patients with pentostam, the peripheral blood lymphocytes and lymph node homing cells proliferate in response to mitogens and Leishmania specific antigens, with increased production of IFN-gamma. Recently it was shown that variation in the IFNGR1 receptor is significantly associated with the risk of developing PKDL.

DIAGNOSIS

Diagnosis of PKDL is based on clinical presentation and history of VL and pentostam treatment. Microscopic demonstration of the parasite in lesions has low sensitivity (20–30%), but this increases to 80–90% using molecular biology and immuno-histochemistry techniques. Serological diagnosis is of limited value in endemic areas, however IgG1 is elevated in the sera of PKDL patients. PKDL sera have been found to consistently react with antigens of 110 and 65 KDa in Leishmania lysate. Isolation of the parasites from PKDL lesions is rather tedious and prone to contamination, but can be achieved by primary culture of skin slits in NNN media with frequent subculture in the same medium, even when
apparent parasite growth is not observed. Parasite culture allows isoenzyme typing and more detailed epidemiological analysis.

TREATMENT

Most PKDL patients in eastern Sudan heal spontaneously and don’t need medical interference. However, PKDL patients with severe or persistent skin lesions might need treatment with a prolonged course of pentostam at 20mg/kg for 2–3 months. Alternative treatment with pentamidine and ketoconazole has been used in areas that report parasite resistance to pentostam. A trial for immunotherapeutic treatment of persistent PKDL lesions with alum-precipitated Leishmania major plus BCG has started in Sudan (personal communication).

CONTROL

PKDL has been thought to contribute to the spread of VL infection by serving as a source of parasites; the presence of PKDL patients has been associated with the occurrence of VL epidemics in India, where VL transmission is thought to be anthropotonic. However, though it has been suggested that PKDL plays a role in transmission of Leishmania parasites in Sudan, this has not yet been proved. Since the same parasite zymodemes (L. donovani MON 18; L. infantum MON30, MON 267; L. archibaldi MON 82) were reported in PKDL patients and animal reservoirs, the transmission of these isolates could be zoonotic, while other zymodemes that were reported only in humans and not in animal reservoirs could be transmitted anthropotonically, with PKDL possibly playing the role of parasite source.

Possible variation in the sensitivity to pentostam treatment of some isolates in eastern Sudan might contribute to the persistence of the parasite after completion of the treatment course.

References


4.6 EPIDEMIOLOGY OF LEISHMANIA-HIV CO-INFECTIONS

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Visceral leishmaniasis (VL) in Mediterranean countries is caused by *Leishmania infantum*, which appears as a hypoendemic disease with 0.2-0.3 cases every 100,000 inhabitants. VL cases associated with HIV infection were increasingly reported between 1985 and 1997, when the introduction of highly active antiretroviral therapy (HAART) drastically reduced the number of co-infection cases seen. The dog acts as a reservoir, and the prevalence of canine leishmaniasis is 5-20% or even more. The sandflies responsible for transmission are: *Phlebotomus perniciosus*, *P. ariasi*, *P. perfiliewi* and *P. neglectus*, from west to east respectively.

Possible overlap in transmission of any *Leishmania* species (which normally is rural and periurban) with HIV transmission (which is urban and periurban) is a problem in those countries where both infections are highly endemic, such as India, Sudan, and Brazil. This situation becomes more acute with the now-established ruralization of HIV transmission and the urbanization of *Leishmania* due to population movements. However, the great majority of *Leishmania*/HIV co-infection cases are still reported in southern Europe; Spain has the great majority of cases, followed by Italy and France. So far, few cases are reported from the above-mentioned countries in which 90% of all VL normally occurs.

Visceral leishmaniasis in immunocompetent individuals in Mediterranean countries was basically found in children until the mid eighties, but recently the age pattern has changed. The AIDS pandemic does not seem to be the only reason for this; in southern France, fewer than half the adult VL patients are currently HIV+, although in Spain the proportion is higher. The prevalence of co-infection has been established: 7-17% of febrile HIV+ and 4% of non-febrile HIV+ subjects (121 patients in total) had amastigotes in bone marrow. In fact, the WHO estimates that 2-9% of all AIDS patients in southern Europe had developed VL before the HAART era.

In southern European countries, the mean age of co-infected patients is 29-33 years, very similar to the age at which HIV infection is more frequent; 80-85% of co-infected patients are male, as occurs in HIV infection also. There are two main concepts about the HIV infection pattern, which is quite similar in most South European countries where co-infection occurs: the AIDS incidence in South European countries is higher than the European average, being almost four-fold higher in Spain; and the number of drug addicts per million inhabitants in Spain is 5-7 times greater than the mean for European countries, and even 1.6 times greater than Italy. When risk groups for HIV infection are analysed in Spain, intravenous drug addicts (IVDU) represent 66% of the total, with homosexuals (15%), heterosexuals (7%), hemoderivatives (2%) and hemoderivatives and unknown (10%) being far behind them. The cases of co-infection are distributed as follows: IVDU represent 50-92%, sexual transmission (homosexual, bi- or heterosexual) 5-40%, hemoderivatives 4-13%, and other unknown causes 3%. The frequency of co-infection among IVDU is thus 15-25% greater than the HIV frequency for the same risk group.

The question always raised in co-infection is whether it represents a latent infection reactivated by immune depression or whether it is a primary *Leishmania* infection taking advantage of the lymphocyte reduction caused by HIV infection. It is estimated that only one in every five or ten immunocompetent persons infected by *Leishmania* subsequently suffers from clinical VL. The *Leishmania* infection/disease ratio in HIV+ patients is unknown, but it seems evident that all parasitized and severely immunodepressed patients will develop symptomatic disease.

There are two hypotheses to explain co-infection. The first is based on the fact that intradermal skin-positive subjects may be asymptomatic carriers of *L. infantum* which, during a later immunodepression, would develop into clinical VL. In this way, the HIV infection would have an unmasking effect on the true endemity of *Leishmania* infection. In the case of HIV infection, it is to be supposed that reactivation is also a matter of fact.

The second possibility is that, since the need for a T lymphocyte-mediated immune response to control *Leishmania* infection is known, the parasite takes advantage of the existing immunodepression at the time of primary infection to establish itself and develop into a clinical syndrome. This might also explain the presence of strains which are poorly pathogenic in immunocompetent hosts and even the appearance of non-pathogenic flagellates. This sec-
ond hypothesis takes into account transmission by primary infection. *Leishmania* transmission in HIV+ patients occurs naturally, by infection through the bite of parasitized sandflies, as in the general population, in the rural or periurban environment, which is commonly where intravenous drug addicts share drugs and syringes. However, in light of the epidemiological and microbiological data, we suggest a second cycle complementary to the conventional one, maintained among intravenous drug addicts who share syringes, a risk group which includes up to 66% of all those infected by HIV. This cycle has been defined as *artificial*, since syringes substitute for sandflies and metacyclogenesis is unnecessary as amastigotes are transmitted; as *epidemic*, because of the form of presentation and number of cases; and as *anthroponotic*, since drug addicts act as parasite reservoirs.

The ease with which sandflies can be infected in the laboratory with the blood of co-infected patients suggests that these immunodepressed subjects serve as true secondary leishmaniasis reservoirs in the natural environment. The epidemiological risk which co-infected individuals pose for the general population is remote, even though we were able to demonstrate by xenodiagnosis that all co-infected patients were infective to sandflies in inverse correlation with the CD4+ count.
Annex 5

IMPROVED STRATEGIES AND POLICIES

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5.1 ECONOMIC ASPECTS OF VISCERAL LEISHMANIASIS CONTROL

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Leishmaniasis has recently earned public attention as one of the “most neglected diseases” in the field of drug research and development. It is indeed one of those diseases for which we lack effective, affordable, and easy to use drugs, and the pharmaceutical industry has few incentives to engage in their development (Yamey & Torreele, 2002).

The term “neglected” can easily be extended from research to control. At country level, the leishmaniases are often a hidden problem; patients live in remote areas with poor access to services and case loads are poorly documented. Desjeux (UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 1997) puts it this way “Affected classes of population are those of lowest socio-economic status, who have minimal political power to influence the decisions of the government and a very limited capacity to assume the costs of the disease.”

At international level, neglected diseases lack visibility and the leishmaniases are no exception. Until their emergence as a co-infection of HIV/AIDS, they were not perceived as a direct threat to industrialized countries. The importance of the disease as a hindrance for economic development in the South is still not fully recognized, though insiders have little doubt that leishmaniasis is a poverty-related disease.

So far, the field of leishmaniasis control has had very little input from health economists. A few clinical trial reports include pharmaco-epidemiological data (on drug and care costs) and a few cost-effectiveness studies of competing control strategies have been published. This paper reviews the available information on economic aspects of leishmaniasis control, with an emphasis on the visceral form of the disease, and gives a brief outline of priority research questions in the economic field.

BURDEN OF DISEASE

Leishmaniasis patients often have poor access to the health system, with gross underreporting of cases as a result. Therefore, it is a hazardous exercise to estimate the current number of new cases and the proportion of all cases who get proper treatment. WHO/TDR estimates the burden of disease caused by the leishmaniases at 2 357 000 DALYs\(^1\) per year and 59 000 deaths annually. Leishmaniasis is endemic in 88 countries on four continents. More than 90% of cutaneous leishmaniasis (CL) cases occur in Iran, Afghanistan, Syria, Saudi Arabia, Brazil and Peru. More than 90% of visceral leishmaniasis (VL) cases occur in India, Bangladesh, Brazil, and

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\(^1\) DALY: disability-adjusted life-year i.e. the number of healthy years of life lost to premature death and disability
As VL has a very focal distribution, the global figures do not reflect the real impact VL has in affected communities. VL is a deadly disease if left untreated, and can have disastrous consequences when it strikes a non-immune population. In the Sudan, a VL epidemic caused major disruption of a famine-stricken society when an estimated 100,000 persons died between 1984 and 1994 in Western Upper Nile Province (Seaman et al., 1996). Reported incidence rates of kala azar in endemic communities vary between 2/1000 person-years in Kenya (Schaefer et al., 1995), to 14/1000 person-years in Ethiopia (Ali & Ashford, 1994), to, in a community in eastern Sudan, an incidence rate of 40/1000 person-years (Zijlstra et al., 1994).

COST-OF-ILLNESS STUDIES

Despite the considerable burden of disease caused by the leishmaniasises, there has been little attempt to quantify the economic implications of the disease in affected communities. Dedet et al. (1991) studied the socioeconomic cost of cutaneous leishmaniasis in French Guyana where it is considered a serious hindrance for economic development of the forest area. The authors estimated the direct costs for health services related to the care of 175 patients in 1979–1980 at FF 3216 per case (approximately US$ 480 per case). Interestingly, hospitalization costs accounted for 82% of the total cost, more related to the need for supervised administration of the relatively toxic drugs (meglumine antimoniate and pentamidine) than to the condition itself. Wijeyaratne et al. (1994) have drawn attention to the tremendous barrier the visceral form of the disease (VL) poses to the development efforts of affected communities, without quantifying their observation. In every member of a family where a VL case occurs, many days of productive life are lost due to this severely debilitating disease. Thakur (2000) described the socioeconomic conditions of a cohort of 938 patients of parasitologically confirmed VL from Bihar in India. Seventy-five per cent of those patients were classified as poor (daily income < US$ 1) and 82% were engaged in agriculture and/or animal husbandry. Seventy-seven per cent lived in mud or grass covered houses. Delays between onset of disease and medical care seeking were considerable, as only 33% of patients attended within 1 month of onset.

Adhikari & Maskay (2003) discuss in a letter to the editor an unpublished study of the direct and indirect costs related to kala azar for households in two districts of Nepal. The analysis suggested that the total cost (US$ 210) to a sampled household of a kala azar episode consumed almost two and a half times an average annual per capita household income (US$ 82). Fourteen out of 18 kala azar affected households fell below the absolute poverty line (US$ 78/inhabitant/year) with the other 4 not far behind. The results of this household survey indicate that the poor are especially susceptible to the disease and that disease-related expenditure was catastrophic.

An Indian health economist recently looked into the household costs related to kala azar by surveying kala azar patients in villages in Bihar (n= 31) and West Bengal (n= 37) (Annigeri, unpublished report). Mean annual income of patients was US$ 439.2 and 191.2 respectively, while their mean expenditure on the kala azar episode was US$ 401.6 and 59.8 respectively. The differences between the states could be related to observed differences in duration of illness and in care-seeking behaviour of patients. Both might be explained by differences in accessibility of the health system. Annigeri relates that Bihar patients had to borrow 47 per cent and West Bengal patients 81 per cent of total treatment expenditure.

COST-EFFECTIVENESS ANALYSIS OF COMPETING CONTROL STRATEGIES

A few studies have addressed the choice of most cost-effective strategy for VL control. The first published cost-effectiveness analysis (CEA) study tried to answer the question of whether VL control was cost-effective in an emergency setting in comparison to other relief measures (e.g. diarrhoea control). Griekspoor et al. (1999) estimated the cost-effectiveness ratio of VL treatment in a relief programme in Sudan at US$ 18.40 per DALY averted (uncertainty range between US$ 13.53 and US$ 27.63), and US$ 595 per life saved. The average cost of this programme was US$ 394 per VL patient treated. As
any health intervention costing less than US$ 25 per DALY averted is considered ‘very good value for money’ by the World Bank, the authors concluded that in the local conditions, their programme was cost-effective. Calculations were based on the use of branded pentostam, at an average cost of US$ 100 per patient. Since that time, the nongovernmental organization has switched to generic antimonials, which reduce the drug cost by a factor 5.

In 1999, for an M.Sc thesis in economics, S. Pokhrel of Chulalongkorn University in Thailand compared patient and provider costs related to two alternative programmes: outreach case detection, and health facility based detection. Patients’ costs were assessed by interviewing a sample of 50 VL cases detected by the two programmes in 1998–99. Median costs to patients in the outreach programme were found to be US$ 25, but were six times higher in the health facility based programme (US$ 146). Indirect costs due to absence from work accounted for more than half the costs in both programmes. The study showed that the outreach programme detected 40.9 cases per 100,000 population at risk while the health facility based programme detected 34.1 cases. The cost per case detected in the outreach programme was US$ 124 and in the health facility based programme it was US$ 191. The outreach and health facility based programmes incurred US$ 131 and US$ 200 respectively for aversion of one death.

Boelaert et al. (1999) used CEA to address the question of most appropriate diagnostic test strategy for VL if splenic aspirates are not feasible. The following strategies were compared: treatment of all suspects on clinical grounds (strategy A); parasitological investigation of bone marrow aspirate (strategy B); two-step testing by means of the direct agglutination test (DAT) followed by treatment of patients with high titres as well as those with parasitologically confirmed borderline titres (strategy C); and a single serological test, the DAT (strategy D). The results for each strategy were expressed in cost in US$ per life saved. The most effective strategy in terms of number of lives saved was strategy A (treating all suspects). The effectiveness of strategies C and D was close to that of strategy A and far better than that of strategy B. Diagnosis by parasitological investigation (strategy B) was the most cost-effective strategy at US$ 448 per life saved. As the cost-effectiveness ratio of serological diagnosis (US$ 465 per life saved) was not substantially higher than that of parasitological diagnosis (incremental cost-effectiveness ratio US$ 491 per life saved), the authors recommended serology because of its higher effectiveness. Though the baseline analysis was prepared using the cost of branded antimonials (Pentostam®), the conclusion remained the same when prices of generic drugs were used instead. The overall cost of test-treatment strategies was much more dependent on the cost of treatment than on the cost of testing. In a subsequent paper, the same authors performed a further sensitivity analysis including a variety of scenarios for potential improvements in drug therapy, which did not alter the above conclusion (Boelaert et al., 2002). Whenever splenic aspiration is not feasible, a serological test-treatment strategy remains the optimal choice, even when better drugs become available.

Recently a number of therapeutic breakthroughs have occurred, such as the adoption of generic antimonials for VL control in East Africa (Veeken et al., 2000), the clinical development of miltefosine as the first oral drug for VL (Sundar et al., 2002), the evaluation of low-cost formulations of lipid formulations of amphotericin B (Sundar et al., 2004), and the evaluation of paromomycin in combination therapy with short-course sodium stibogluconate (Thakur et al., 2000b). So far, only limited cost data are available to compare regimens; a formal CEA could facilitate the decision on optimal therapeutic regimen. See Table 1 for an overview by V. Vanlerbergh et al. of published cost and efficacy estimates of available drug regimens, most of them obtained in clinical trials. The table shows that efficacy values are quite comparable between the different regimens, hence the importance of careful costing of drugs and related care. The mode and duration of administration play a crucial role, as the cost of care has a huge impact on the overall cost-effectiveness ratios. In this respect, we should be very cautious, as the costing of patient care was carried out in hospital settings and this may not reflect the reality of a first-line health service. Also, effectiveness in real-life conditions might be quite different from efficacy obtained in clinical trials, and moreover, compliance and thus effectiveness is dependent on duration of regimen. Last but not least, the data in the table apply only to HIV-negative patients, and the effectiveness of VL drugs in HIV co-infected patients is not at all comparable.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Regimen</th>
<th>Total dose (mg/kg)</th>
<th>Efficacy (%) (range)</th>
<th>Drug cost (US$) (range)</th>
<th>Care cost (US$) (range)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium stibogluconate</td>
<td>20 MKD for 30 days IM(^5)</td>
<td>600</td>
<td>92% (36–96%)</td>
<td>22 (22–200)</td>
<td>112 (112–359)</td>
<td>Seaman et al., 1993; Griekspoor et al., 1999; Veeken et al., 2000; Sundar et al., 2000; Thakur et al., 2000; Ritmeijer et al., 2001; Murray, 2000; Murray, 2001</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>1 MKD, 15 infusions on alternate days(^6)</td>
<td>15</td>
<td>97% (96–99%)</td>
<td>90 (90–220)</td>
<td>240 (240–370)</td>
<td>Sundar et al., 2002a; Sundar et al., 2004; Thakur et al., 1999; Sundar et al., 2001; Sundar et al., 1997; Murray, 2000</td>
</tr>
<tr>
<td>Lipid formulations of amphotericin B</td>
<td>2 MKD, 5 days</td>
<td>10</td>
<td>92% (90–96%)</td>
<td>500 (500–1880)</td>
<td>82 (82–95)</td>
<td>Sundar et al., 1997; Sundar et al., 2004; Murray, 2000; Sundar et al., 2000</td>
</tr>
<tr>
<td>(Ambisome(^6), Abelcet(^6))</td>
<td>5 mg/kg, single infusion</td>
<td>5</td>
<td>91% (70–91%)</td>
<td>250</td>
<td>40</td>
<td>Sundar et al., 2001; Sundar et al., 1998</td>
</tr>
<tr>
<td>Various regimens</td>
<td>5–20</td>
<td>(78–100%)</td>
<td>(250–3760)</td>
<td>(82–102)</td>
<td></td>
<td>Sundar et al., 2002(b); Sundar et al., 2001; Sundar et al., 1998; Sundar et al., 1997; Murray, 2000; Sundar et al., 2004; Syriopoulou et al., 2003</td>
</tr>
<tr>
<td>FDA: 21 mg/kg given on 7 days over 21 days period</td>
<td>21</td>
<td>100%</td>
<td>1050 (1050–3948)</td>
<td>82 (82–102)</td>
<td></td>
<td>Meyerhoff, 1999</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>2.5 MKD, per os, 28 days</td>
<td>70</td>
<td>94% (88–94%)</td>
<td>110 (110–283)</td>
<td>35 (35–480)</td>
<td>Sundar et al., 2002(a); Sundar et al., 2003; Bhattacharya et al., 2004</td>
</tr>
<tr>
<td>SSG/paromomycin</td>
<td>PM 12 MKD + SSG 20 MKD, 21 days IM(^a)</td>
<td>PM 252+ SSG 420</td>
<td>92%</td>
<td>42 (42–72)</td>
<td>75 (75–244)</td>
<td>Thakur et al., 2000; Murray, 2001; Murray, 2000</td>
</tr>
<tr>
<td>Other regimens</td>
<td></td>
<td></td>
<td>90–94%</td>
<td></td>
<td></td>
<td>Seaman et al., 1993; Thakur et al., 2000</td>
</tr>
</tbody>
</table>

MKD = mg/kg/day; SSG = Sodium stibogluconate; PM = paromomycin; IM = intramuscular; FDA = Food and Drug Administration

\(^1\) Efficacy estimated as proportion of cases with negative parasitology/no clinical signs at end of six months follow-up period

\(^2\) All cost values computed for a 50 kg patient

\(^3\) In the cost of care is included: cost of a hospitalization day (4 US$), doctor’s fees, laboratory costs, infusion sets, syringes/needles, adjuvant treatment. Based on costing data of India/Nepal (S. Rijal, personal communication)

\(^4\) alternatively: intravenous (IV), 28 days

\(^5\) alternatively: 1 MKD, 20 daily infusions

CONCLUSION: SOCIOECONOMIC RESEARCH QUESTIONS IN THE AREA OF VISCERAL LEISHMANIASIS CONTROL

To support control efforts and to argue the case for greater investment in this disease, there is a true need for undertaking socioeconomic studies. We suggest that the following issues should be tackled:

• A more accurate estimate of the burden of leishmaniasis and of disease trends is required for reasons of advocacy as well as management of control efforts. The fact that the disease is so under-reported and under-recognized has hindered its profile at both national and international level; it has also prevented health delivery systems in affected countries from allocating the appropriate resources for disease control. Control efforts will not become coordinated until proper surveillance data are fed into the management cycle; in this respect, a regional approach to surveillance might well be justified in some endemic areas, as disease foci are often situated across borders.

• Micro-economic studies (household costs/cost-of-illness studies) should demonstrate much more clearly that leishmaniasis is a poverty-related disease, and how it impacts on development. The millennium goals with their emphasis on poverty reduction provide a unique window of opportunity to raise awareness on the neglected diseases at international level. Whereas in the past, ill-health was seen as the consequence of poverty, there is growing consensus amongst macro-economists that disease in fact is a root cause of poverty (Canning, 2003). Hence the strategic importance of research to demonstrate (the obvious): the link between leishmaniasis and poverty.

• Cost-effectiveness analysis of competing choices in VL control strategies could provide welcome advice in difficult choices on resource allocation within control programmes. The few studies we retrieved discuss competing choices for test-treatment of VL, which is indeed the principal control strategy. One of the main limitations for CEA in this respect is that reliable cost data are lacking. The published clinical trials include some cost data, but patient care costs in tertiary care centres cannot be extrapolated to district health systems. As the optimal choice of drug regimen is so sensitive to patient care costs, it is important to elucidate this question. These CEA analyses should not be limited to the choice of a drug regimen or the choice of a diagnostic test, but relate to the overall context of the health system in which case management has to take place. Furthermore, if the efficiency of alternative methods of vector control and control of the animal reservoir was better known, this might provide leverage for intensification of leishmaniasis control efforts.

If we want to translate the substantial gains in scientific knowledge and tools for leishmaniasis control achieved over the past decade into demonstrable changes in disease burden, it seems necessary to invest in more accurate monitoring of disease trends, in better advocacy for disease control within the global aim of poverty reduction, and in more rational allocation of resources to the most cost-effective control strategies.

References


Ritmeijer K et al. Ethiopian visceral leishmaniasis: generic and proprietary sodium stibogluconate are equivalent; HIV co-infected patients have a poor outcome. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 2001, 95:668–672.


5.2 CLIMATE VARIABILITY AND VISCERAL LEISHMANIASIS IN EUROPE

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INTRODUCTION

A three-year pan-European project entitled Climate change and adaptation strategies for human health (cCASHh), funded under the European Union thematic programme on Energy, Environment and Sustainable Development (EESD-1999), began in 2000. The aim of this project was to provide a scientific basis for the development within the health sector of response strategies to climate variability and change. The project included academic partners in the United Kingdom, Sweden, the Netherlands, Germany, Italy, and the Czech Republic.

This paper reports the findings of a review of impact of climate variability and change on leishmaniasis, and the results of discussions at a meeting on vector-borne diseases and climate change held in Prague, 5–7 May 2003. A literature search was conducted to answer questions about the current distribution of visceral leishmaniasis (VL) in the WHO European Region, the influence of environmental and climatic factors on disease risk, the possible impacts of future climate, and future research needs.

CURRENT DISTRIBUTION AND RISKS

In Europe, VL is considered to be a rare disease, although its incidence increased significantly in the region during the 1990s. This was due in part to the large proportion of Leishmania/HIV co-infections (approximately 25%–70% of adult VL cases are co-infected with HIV) and to better reporting after the establishment of a WHO surveillance network in 1994 (Desjeux et al., 2000). Non-HIV VL incidence rates have increased in Italy and France (including a four-fold increase in Alpes-Maritimes), and new endemic areas have been detected where no previous autochthonous cases had been reported, e.g. in northern Italy, North Croatia, Switzerland and Germany.

The distribution of leishmaniasis worldwide is limited by the distribution of its vector sandfly and by the latter’s susceptibility to cold climate, ability to support internal development of specific species of Leishmania, and tendency to take blood from animals or humans only (WHO, 2000[b]). Populations of Phlebotomus perniciosus, one of the known vectors of Mediterranean VL, have been found as far north as Paris (Parrot, 1922), and recently also in Gerweiler in Germany (Naucke & Schmitt, 2004). Table 1 lists the distribution of the different species that may act as vector for L. infantum, the pathogen that causes VL in Europe.

Since the mid-1990s, the worldwide geographical distribution of endemic leishmaniasis has expanded (WHO, 2000[a]). This spread is probably due to a combination of factors, among them increased monitoring, intensified research, demographic change,

<table>
<thead>
<tr>
<th>Vector</th>
<th>Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. ariasi</td>
<td>France, Italy, Portugal, Spain</td>
</tr>
<tr>
<td>P. neglectus</td>
<td>Albania, Greece, Italy, Malta, Romania, former Socialist Republic of Yugoslavia, southern Austria</td>
</tr>
<tr>
<td>P. perifiliei</td>
<td>Albania, Greece, Italy, former Socialist Republic of Yugoslavia</td>
</tr>
<tr>
<td>P. perniciosus</td>
<td>France (incl. Corsica), Germany, Italy, Malta, Portugal, Spain, southern Austria</td>
</tr>
<tr>
<td>P. tobbi</td>
<td>Albania, Cyprus, Greece, Italy (Sicily), former Socialist Republic of Yugoslavia</td>
</tr>
<tr>
<td>P. mascitti</td>
<td>Belgium, Germany, France (incl. Corsica), Italy, Monaco, former Socialist Republic of Yugoslavia, Spain, Switzerland</td>
</tr>
</tbody>
</table>

land-use/land cover changes that create new habitats and/or changes in microclimate, and changes in seasonal climate. The overlap between geographical areas with high risk of both leishmaniasis and HIV is increasing with the spread of leishmaniasis (typically a rural disease) into urban areas and the increased spread of HIV into rural areas (WHO, 2000[a]).

Within the European Region, cases of VL have been reported from Albania, Bosnia & Herzegovina, Bulgaria, Croatia, France, Greece, Hungary, Italy, Macedonia, Malta, Monaco, Portugal, Romania, Spain, and Yugoslavia. Leishmaniasis is also transmitted in the adjoining countries of Azerbaijan, Cyprus, Georgia, Kazakhstan, Tajikistan, Turkey, Turkmenistan and Uzbekistan. Figure 1 shows the regional distribution of VL compared to cutaneous leishmaniasis (CL).

Most cases of co-infection in Europe are reported from the most densely populated areas and provinces and, as shown in Figure 2, there is a predominance of cases in coastal areas (75%). In south-western Europe, 80% of co-infection cases are from urban areas, the main cities being Lisbon and Porto in Portugal; Barcelona, Granada, Madrid and Seville in Spain; Marseille and Nice in France; and

![Figure 1. Approximate distribution in Europe of visceral leishmaniasis and its vectors compared to cutaneous leishmaniasis and its vectors](image1)

Note: Cases of VL reported from the Paris metropolitan area were probably the result of infection elsewhere.

![Figure 2. Cases of Leishmania/HIV co-infection per locality and population density, 1990–1998.](image2)

Modified from Desjeux et al., 2000
Genoa, Milan and Catania (Sicily) in Italy. This distribution pattern, however, is partly related to the current location of the WHO surveillance centres (which specifically monitor *Leishmania*/HIV co-infections) rather than to other factors. Most of the centres are located near cities like Rome and Catania in Italy; Madrid, Barcelona, Seville, Bilbao, Granada and Palma de Mallorca in Spain; Paris, Marseille, Montpellier and Nice in France; and Lisbon in Portugal.

Populations at risk include people living in rural and periurban areas where both sandflies and reservoir animals are prevalent. VL used to be found predominantly in children but in recent years an increasing proportion of adult cases (non-HIV) has been reported. This change in age distribution is probably caused by several factors, such as changes in human exposure patterns, environmental changes, and improvements in case diagnosis and notification. Improvements in nutritional and general health status in European children have probably played a roll in reducing the characteristic high susceptibility of children to this disease.

The sandfly vector is mainly active during the night, and the highest risk for contracting the disease from sandfly bites is therefore between dusk and dawn.

Urban areas have become high-risk locations of late. High-risk populations in urban environments are people who are HIV-positive. In Europe, 77% of *Leishmania*/HIV co-infected patients are aged between 31 and 50 years, and 83% of them are men (WHO, 1997). Another risk group is intravenous drug users who share syringes (Cruz et al., 2002; Pineda et al., 2002; Amela et al., 1996). The highest risk for VL is found among HIV-infected intravenous drug users, who account for 71% of co-infection cases (Desjeux et al., 2000).

In addition, *Leishmania* can be transferred through blood transfusion (Kubar et al., 1997); the trans-placental route of infection is also possible (Meinecke et al., 1999).

*Leishmania*/HIV co-infections accelerate and aggravate both leishmaniasis and AIDS symptoms (Desjeux et al., 2000). Also, relapses of leishmaniasis after treatment are common among persons with HIV co-infection. The mean survival of co-infected patients is only 13 months (WHO, 2000[b]). The current number of HIV-infected persons in western and eastern Europe (not including the Russian Federation) is approximately 580 000 (WHO, 2001). In the Mediterranean basin, 1.5%–9% of AIDS patients develop VL (WHO, 2000[a]).

Most of the co-infection cases have been reported from France, Italy, Portugal and Spain, but co-infection is prevalent in Albania, Croatia and Greece as well. On average, the number of reported cases of *Leishmania*/HIV co-infection increased during the late 1990s relative to the number of reported HIV cases in the same period (Desjeux et al., 2000). However, the number of cases of co-infection has recently started to fall in southern Europe owing to the use of new therapeutic methods, i.e. highly active antiretroviral therapies (HAARTs) (Table 2).

### INFLUENCE OF ENVIRONMENTAL AND CLIMATIC FACTORS ON DISEASE RISK

The distribution of VL in Europe is significantly less than the distribution of the sandfly vectors. The occurrence of disease transmission within the range of the vectors depends on vector abundance, vector survival, vector biting rate (i.e. gonotrophic cycle), the extrinsic incubation period, and the length of the transmission season. Each of these parameters is climate dependent, but the precise relationship with climate needs to be further studied and evaluated. Caution is also required in the interpretation of laboratory experiments as sandflies are able to evade extreme weather conditions in the field by their choice of resting site and time of activity.

Temperature and humidity are the two most important climatic factors for sandfly survival, development and activity. *Phlebotomus* sandflies can survive cold temperatures in diapause (overwintering), which is initiated by a combination of low temperature and reduced daylight and can last 4 to 8 months depending on location. In Europe, the biting activity of sandflies is strongly seasonal, and restricted to the summer months in most areas. Adult activity as well as larval development slows down considerably below 20°C. However, some species are active even at much lower temperatures, e.g. *P. neglectus* in Greece at 13°C and *P. mascitti* in Germany at 13.5°C (Naucke, 1998; Schmitt, 2002).

**Table 2.** Number of reported cases of VL/HIV co-infection in southern Europe, 1990–2001

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>France</td>
<td>127</td>
<td>132</td>
<td>59</td>
</tr>
<tr>
<td>Italy</td>
<td>144</td>
<td>85</td>
<td>106</td>
</tr>
<tr>
<td>Portugal</td>
<td>29</td>
<td>88</td>
<td>42</td>
</tr>
<tr>
<td>Spain</td>
<td>473</td>
<td>412</td>
<td>214</td>
</tr>
</tbody>
</table>
Temperature also affects the parasite itself; evidence indicates that *L. infantum* is prevalent within the 5–10°C January and 20–30°C July isotherms, usually located below latitude 45 N and below 400–600 m above sea level (Kuhn, 1999). The worldwide distribution of sandflies is considered to be confined to areas that have at least one month with a mean temperature of 20°C (WHO, 1984). However, it has been suggested that the limit of the European distribution of vectors and parasites better corresponds to the 10°C annual isotherm (Maier et al., 2003). This is in accordance with recent findings of vectors and isolated autochthonous cases of leishmaniasis in southern Germany, where the July isotherm is 16°C to 18°C. The highest focus of leishmaniasis in Europe is the Guadix focus (Andalusia, Spain), at an altitude of 900 to 950 m above sea level.

Sandflies are sensitive to sudden temperature changes and usually prefer regions with small differences between the maximum and minimum temperature. Sandfly survival can be reduced if the climate gets too hot and dry, even though the flies may rest in cold, humid places during the daytime (McCarthy et al., 2001). The resting sites of adults are known for a few species of sylvatic sandfly; they include tree holes and trunks. Peridomestic species rest on walls and, at hot times of the day, retreat into cracks and crevices (WHO, 1984). Poroton stone buildings, for example, have the ability to store humidity during the night and evaporate it during the day, producing favourable conditions for adult sandflies to survive the hot, dry summer days.

In addition to the direct association between climate and leishmaniasis transmission, climate has indirect impacts by influencing (1) the distribution of hosts; (2) the local vegetation (important as resting sites and sugar sources); and (3) the patterns of human exposure to sandfly vectors.

### POSSIBLE IMPACT OF FUTURE CLIMATE RISKS

With climate change, the distribution range of both the sandfly vector and the pathogen may extend northwards and into higher altitudes (WHO, 1999). In currently endemic areas, higher seasonal temperatures would lead to prolonged activity periods and shorter diapause periods. This could result in an increased number of sandfly generations per year. In addition, higher temperatures are likely to accelerate maturation of the protozoan parasite, thereby increasing the risk of infection (McCarthy et al., 2001; Rioux et al., 1985). However, if the climate becomes too hot and dry for the vector to survive, the disease may disappear from some localities even though the vector may adapt by resting in cool, humid places during the daytime.

Imported infected dogs can contribute to the emergence of leishmaniasis in new locations. They are a potential source of the pathogen if the vector expands its geographical distribution due to change in climate (WHO, 1999). Several imported cases of canine leishmaniasis are, for example, reported from Germany (Gothe et al., 1997) and the Netherlands (Slappendel, 1988) every year.

In order to develop more sophisticated climate–vector–disease scenario models, increased knowledge is needed on the short- and long-term effects of climate variation on leishmaniasis risk. This can be achieved in several ways: (1) experimental evidence

#### Table 3. Important *Phlebotomus* species in Europe and their climate thresholds

<table>
<thead>
<tr>
<th>Vector</th>
<th>Minimum temperature (larvae)</th>
<th>Optimum temperature (larvae)</th>
<th>Maximum temperature (larvae)</th>
<th>Preferred humidity (adults)</th>
<th>Preferred altitude</th>
<th>Causative organism</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. perniciosus</em></td>
<td>0°C</td>
<td>25°C</td>
<td></td>
<td></td>
<td>&lt;900 m</td>
<td><em>L. infantum</em> ^a^</td>
</tr>
<tr>
<td><em>P. perfiliewi</em></td>
<td>-4°C</td>
<td>25°C</td>
<td>33°C</td>
<td>60–80%</td>
<td>&lt;450 m</td>
<td><em>L. infantum</em> ^a^</td>
</tr>
<tr>
<td><em>P. ariasi</em></td>
<td>5°C ^b^</td>
<td>30°C ^b^</td>
<td></td>
<td></td>
<td>300–500 m ^c^</td>
<td><em>L. infantum</em></td>
</tr>
<tr>
<td><em>P. neglectus</em></td>
<td>-4°C</td>
<td>25°C</td>
<td>30°C</td>
<td>60–80%</td>
<td>300–1000 m</td>
<td><em>L. infantum</em> ^a^</td>
</tr>
<tr>
<td><em>P. papatasi</em></td>
<td>5°C</td>
<td>-4°C (Greece)</td>
<td>28–34°C ^c^</td>
<td>35°C</td>
<td>36–45% ^c^</td>
<td>0–1000 m</td>
</tr>
<tr>
<td><em>P. sergenti</em></td>
<td>31–33°C ^c^</td>
<td></td>
<td>0–45% ^c^</td>
<td></td>
<td></td>
<td><em>L. tropica</em> ^b^</td>
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<tr>
<td><em>P. mascetti</em> ^d^</td>
<td>-4°C</td>
<td>19°C</td>
<td>28°C</td>
<td>60–80%</td>
<td>0–400 m</td>
<td></td>
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</table>

measuring the influence of temperature or humidity (for example) on sandfly or Leishmania biology; (2) field evidence that sandfly biological parameters vary with climate; (3) field evidence that geographic variation in sandfly abundance (including presence vs. absence) or Leishmania infection rate correlates with climatic variables; and (4) field evidence that temporal variation in sandfly abundance or Leishmania infection rate (including seasonal and long-term trends) correlates with climatic variables. As different sandfly species are responsible for Leishmania transmission across Europe, climate change will have different impacts on VL risk in different parts of the region.

Regional climate models for leishmaniasis should therefore be constructed according to the local vector species.

FUTURE RESEARCH NEEDS

There is a need for further research on visceral leishmaniasis and its vectors in Europe. Better understanding of the current situation would allow more specific risk evaluation and form the basis for predicting change in distribution and endemicity due to environmental and climatic changes in different parts of Europe. Some main issues are to:

- Collate available data on sandfly and parasite distribution, and sandfly seasonality, in relation to information and data on climate and environmental variables.
- Collect new field data on sandfly biological parameters (including activity patterns, seasonality, survival, infection rates) in relation to defined climatic and environmental variables – latitude and altitude transects over full seasons. Develop a network of fieldworkers across Europe able to use standard methodology for measuring these parameters (especially infection rate).
- Collect laboratory data on vectorial competence, including extrinsic incubation period and diapause determinants, of different European vector species.
- Collect new field data on dog prevalence, and set up a network of veterinarians across Europe to use standardized methodology.
- Further analysis of HIV-leishmaniasis data.

References


5.3 TOOLS AND STRATEGIES FOR USE OF INSECTICIDE IMPREGNATED FABRICS IN THE CONTROL OF SANDFLIES AND LEISHMANIASIS

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The use of insecticide impregnated fabrics (ITNs) is one of the most effective methods of reducing man-vector contact and intra- and peri-domiciliary transmission of vector-borne diseases. During the past two decades, encouraging results on the use of ITNs against phlebotomine sandflies and leishmaniasis have been obtained from several countries, including Italy (Maroli & Lane, 1989), Burkina Faso (Majori et al., 1989), Syria (Tayeh et al., 1997; Desjeux, 2000), Sudan (Elnaiem, 1994, 1997, 1998; Elnaiem et al., 1999; Elnaiem, Elnahas & Aboud, 1999), Kenya (Mutinga et al., 1992, 1993; Basimike & Mutinga, 1995), Colombia (Alexander et al., 1995) and Venezuela (Feliciangeli et al., 1995; Kroeger et al., 2002). This paper reviews the literature on use of ITNs against sandflies and leishmaniasis and attempts to highlight the remaining gaps in knowledge and the further research needed to be undertaken.

WHAT INSECTICIDE, WHAT DOSE, WHAT FABRIC?

In most studies, the insecticides used have been synthetic pyrethroids, which combine the properties of low to moderate mammalian toxicity (Wells, Grayson & Langer, 1986), low volatility, and high insecticidal activity. Due to their low vapour pressure, the pyrethroid insecticides used for impregnating nets do not repel insects, and therefore the ITNs act as “baited traps”.

The dose of insecticide used to impregnate nets against sandflies depends on the insecticide used, and has varied in the different trials (Alexander & Maroli, 2003). In field experiments, Elnaiem, Elnahas & Aboud (1999) found that only 30 seconds of exposure to bednets impregnated with 10 mg a.i./m² of lambda-cyhalothrin were sufficient to kill 100% of P. orientalis within 1 hour of contact. Maroli & Majori (1991) recorded over 90% mortality within 24 h in P. papatasi and P. perniciosus exposed in the laboratory to mesh treated with 1 g a.i./m²; biting rates were also reduced in both species.

Most studies have utilized polyester or cotton netting material similar to that used for malaria control. Because of their small size however, it is often argued that sandflies can pass through wide-mesh bednets; on the other hand, there is some concern that fine-mesh bednets are not acceptable to potential users in hot climates. Therefore some workers have attempted to test the efficacy of impregnated wide-mesh ITNs. Maroli & Lane (1989) found that permethrin-impregnated nets of 1 cm sq. mesh placed over windows significantly reduced the numbers of P. perfiliewi entering houses in Italy. Similarly, Alexander et al. (1995), Elnaiem et al. (1994), and Elnaiem, Elnahas & Aboud (1999) showed that the sandfly biting rate on human volunteers under impregnated wide-mesh bednets was significantly lower than that under untreated bednets. In contrast to these findings, Feliciangeli et al. (1995) reported that sandflies (mainly Lutzomyia ovallesi) were able to pass through 6 mm mesh curtains treated with deltamethrin at 15 mg/m² and bite volunteers. However, when the mesh size was reduced to 4 mm and the concentration of permethrin raised to 60 mg/m², all sandflies were killed within 30 minutes after 10 minutes of exposure.

EFFECTS OF INSECTICIDE TREATED FABRICS ON ABUNDANCE, INDOOR RESTING, AND BITING RATES OF SANDFLIES

Maroli & Lane (1989) provided the first evidence that impregnated window curtains reduce the number of sandflies entering houses in Italy. Similarly in Burkina Faso, Majori et al. (1989) showed that use of permethrin-impregnated cotton curtains almost completely eliminated the occurrence of endophilic Sergentomyia sandflies. These results were substantiated by Elnaiem et al. (1999), who showed that the biting activity indoors and the resting density of P. papatasi were significantly reduced in rooms fitted with permethrin impregnated curtains as compared to control rooms left without curtains or fitted with non-impregnated curtains; no significant difference was found between the number of nocturnally active sandflies captured on sticky paper traps in the intervention and non-intervention rooms. Interestingly, when nocturnal activity was assessed by hourly night trap collection, it was noticed that most sandflies entering the intervention rooms died immediately after capture. It was therefore concluded that sandflies entering the rooms were not repelled by the permethrin-impregnated curtains but, on entry,
they picked up a lethal dose of the insecticide that prevented their blood-feeding and resulted in subsequent death. This particular study raised concerns about using sticky paper traps to assess the number of sandflies entering rooms fitted with insecticide-impregnated curtains, since this method cannot distinguish between healthy and dying individuals. In contradiction to these findings, Feliciangeli (1995) reported that curtains impregnated with deltamethrin at 60 mg/m² produced no significant reduction in the number of *Lu. ovalelesi* sandflies entering houses in El Ingenio, Mirnada State, Venezuela; the authors attributed this failure to low compliance with the control measure, as discomfort due to high night temperatures frequently prompted local residents to remove the nets from their windows and doors. However, more recently, in Trujillo, Venezuela, Kroeger et al. (2002) reported a significant reduction in the number of *Lu. ovalelesi* and *Lu. youngi* entering houses fitted with lambda-cyhalothrin impregnated curtains, as compared to non-intervention houses fitted with un-impregnated curtains.

Studies on the impact of impregnated bednets have focused mainly on the sandfly biting rate, with little assessment of the impact of nets on sandfly density. In Colombia, Alexander et al. (1995) showed that the sandfly biting rate on volunteers sleeping under impregnated bednets was significantly lower than on those sleeping under untreated bednets. Similar results were obtained by Elnaiem (1994), who found that permethrin impregnated bednets reduced the biting rate of *P. papatasi* from 9.4 to 0.2 bites per night. In a second study, the authors recorded that the number of *P. orientalis* (vector of *L. donovani*) females landing on human collectors without bednets or using untreated nets averaged 32 and 7 per man-night respectively, whereas volunteers using bednets impregnated with lambda-cyhalothrin insecticide received no sandfly bites (Elnaiem, Elnahas & Aboud, 1999).

**EFFECTS OF INSECTICIDE TREATED FABRICS ON THE INCIDENCE OF LEISHMANIASIS**

From the few trials and retrospective studies conducted in different parts of the world, there is strong evidence that impregnated bednets and curtains can significantly reduce the incidence of leishmaniasis (Davies et al., 2003). In a controlled study in Syria, Tayeh et al. (1997) demonstrated a sharp and consistent reduction in the incidence of cutaneous leishmaniasis caused by *L. tropica*. A randomized controlled trial in Kabul, Afghanistan, also showed that permethrin treated nets and chadors (Islamic cloth wraps) each provided 65% protective efficacy against anthroponotic cutaneous leishmaniasis (ACL), whereas lambda-cyhalothrin residual house spraying resulted in 40% protective efficacy (Reyburn et al., 2000). Similarly, in a pilot study in eastern Sudan, Elnaiem (1997, 1998) found that the inhabitants of two villages using bednets impregnated with lambda-cyhalothrin insecticides had significantly less incidence of kala azar as compared to a non-intervention village (1.6% in the intervention vs. 12.4% in the non-intervention village). The study provided the intriguing result that the ratio of clinical to subclinical infection with *L. donovani* changed from 7:1 in the non-intervention village to 1:3 in the intervention villages, probably due to reduction in the transmission of *L. donovani* and/or malaria co-infection. The impact of the ITNs was maintained throughout the three years of the study. However, the trial was limited by lack of additional villages because of their inclusion in a visceral leishmaniasis (VL) vaccine trial. No other studies on the impact of ITNs on VL are reported. However, in a recent case control study in Nepal, people using conventional, untreated, nets were shown to be 70% less likely to develop visceral leishmaniasis (Bern et al., 2000).

The impact of insecticide impregnated curtains on the incidence of cutaneous leishmaniasis was studied thoroughly in a cluster randomized trial in Trujillo, Venezuela, where most transmission takes place within the domestic setting (Kroeger et al., 2002). The authors found that insecticide impregnated curtains provided a high degree of protection against indoor transmission of cutaneous leishmaniasis. After 12 months of intervention using curtains impregnated with lambda-cyhalothrin in 13 sectors of the city, the incidence of leishmaniasis dropped from 4% to 0%. In comparison, the incidence in 13 control sectors, using untreated curtains, rose from 4.5% to 8%.

Recently some studies have been carried out on the protective efficacy of insecticide impregnated clothing, mainly military uniforms. Soto et al. (1995) showed that permethrin-impregnated uniforms reduced the incidence of cutaneous leishmaniasis (CL) in Colombian soldiers on patrol by 75%. These findings, however, were challenged by Asilian et al. (2003), who observed that permethrin impregnation of military uniforms at a dose of 850mg/m² did not significantly protect soldiers in Iran from CL.

**ACCEPTABILITY OF INSECTICIDE TREATED FABRICS, AND COMPLIANCE BY LOCAL POPULATIONS**

Little information has been provided on the accept-
ability and other human behavioural factors affecting ITN use against leishmaniasis. However, some studies have provided valuable information on the efficacy of implementation. In eastern Sudan, for example, a major question was whether bednets are protective against VL since a great deal of man-vector contact takes place outdoors, before people go to bed. However, a socio-behavioural study indicated that the duration of exposure was significantly associated with gender as well as age group (Elnaiem, Elnahas & Aboud, 1999). Impregnated bednets were potentially more protective against *P. orientalis* for women and children than for adult men. Overall, the proportion of people and their duration of exposure to risk of sandfly bites (i.e. after sunset until they went to bed) was 40% unprotected for <1h, 50% unprotected for 1–2h, and 10% unprotected for >2h. Most children in the age groups of less than 5 years and 6–15 years were potentially exposed for <1 h and 1–2 h, respectively. Because VL in Sudan occurs mainly in children, it was concluded that the use of impregnated bednets (outdoors as well as indoors) is highly protective.

**REMAINING GAPS IN KNOWLEDGE AND FUTURE RESEARCH PRIORITIES**

The studies reviewed above clearly show that ITNs are efficacious against sandflies and leishmaniasis. However, further evaluations of impact of ITNs on VL should be encouraged. Furthermore, research on ITNs should move from efficacy studies into evaluation of effectiveness; to do this, more operational research is needed to address the socioeconomic dimensions of ITN use against leishmaniasis and to make knowledge available on practical aspects of bednet intervention. Needed is/are:

- Definition of indicators, for decision-makers, on when to implement ITNs. This can be achieved by strengthening knowledge of sandfly ecology and leishmaniasis epidemiology at regional levels.

- Assessment of financial, managerial, human and material elements needed for large-scale intervention programmes. This can be done through regional-level cost-effectiveness studies.

- Socioeconomic studies addressing behavioural aspects of ITN use, and how to motivate people to use ITNs during leishmaniasis epidemics.

- New research initiatives to encourage the invention of new impregnated fabric tools that can be used outdoors during the early hours of the evening. Lessons from the use of insecticide-impregnated dog collars should lead us to new ideas for controlling human leishmaniasis.

**References**


Annex 6

PARTNERSHIP AND CAPACITY BUILDING

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6.1 THE IMPORTANCE OF RISK FACTORS IN THE CONTROL OF LEISHMANIASIS

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The leishmaniases are vector-borne diseases, and are highly complex: they are usually zoonotic; a large number of *Leishmania* species infect humans; the biological cycles and epidemiological interrelationships between vector, reservoir, humans, climate and ecology are highly variable; they have multifaceted clinical manifestations (due to the genetic heterogeneity of both parasite and humans) and outcomes (which vary from spontaneously cured to not curable) as a consequence of disease.

The magnitude of leishmaniasis has changed little in the last 50 years in some endemic areas of the New World. For instance, in some areas of Peru endemic for cutaneous leishmaniasis (CL), the prevalence during the 1990s was similar to the prevalence before the 1950s (Davies et al., 1994); although the DDT house spraying campaign between the 1950s and 1970s reduced the annual incidence of leishmaniasis, when spraying was stopped around 1972, CL rose again until it had reached its traditional level. The global incidence and prevalence of leishmaniasis increased generally in the Americas between the 1980s and 1990s, despite the intervention measures and efforts of the health authorities. Other vector-borne diseases such as malaria, dengue, yellow fever, and bartonellosis have increased too. The growth of the population, poor levels of hygiene, and increasing periurban poverty are creating favourable habitats for the proliferation of vectors and reservoirs. As a consequence, vector-borne disease such as malaria, dengue, and lymphatic filariasis are becoming major public health problems (Knudsen & Slooft, 1992); the same is now happening with leishmaniasis, which is rapidly becoming urbanized, as reported from Manaus and Belo Horizonte (Profeta da Luz et al., 2001).

The contribution that scientific knowledge is making to New World leishmaniasis is indisputable. A large number of papers have been published since the early 60s, including detailed reviews of parasitological, ecological, entomological, immunopathological, clinical, therapeutic and public health aspects. Before

Table 1 summarizes the intervention targets recommended for leishmaniasis by the WHO Expert Committee [WHO, 1990]. These actions have been applied frequently, partially and independently of each other. The most common intervention is early diagnosis and treatment (action against the parasite), but this does not reduce transmission. The vector is usually controlled (inside houses and in the peridomestic and sylvatic environments) only in outbreaks, with limited benefit in the long term, while control of the animal reservoirs (domestic and wild) is restricted to special situations. Personal protection measures (which reduce man-fly contact) are used only on a small scale and in time-limited situations, usually by tourists or personnel employed by extraction companies working in the jungle. A vaccine is an important alternative, but is still in the experimental stages.

These actions have not so far been successful in controlling leishmaniasis in the New World. The weakness of the strategy is the biomedical approach, the application of interventions through vertical programmes, the lack of sustainability in the long term, and the low or absent participation of people from

<table>
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<th>Table 1. Intervention targets in leishmaniasis</th>
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<tr>
<td><strong>Vector control</strong></td>
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<tr>
<td>Insecticide spraying</td>
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<td>Genetic control</td>
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<td>Biological control</td>
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<tr>
<td>Chemical control</td>
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<tr>
<td><strong>Control of animal reservoirs (domestic and wild)</strong></td>
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<tr>
<td>Drugs</td>
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<tr>
<td>Vaccine</td>
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<tr>
<td><strong>Personal protection</strong></td>
</tr>
<tr>
<td>Vaccine</td>
</tr>
<tr>
<td>Repellents</td>
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<tr>
<td><strong>Early diagnosis and treatment</strong></td>
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endemic areas. The approach using risk factors is an alternative to this classical approach.

In the Latin American literature, several factors have been associated with a high risk of transmission. These include occupational activities mainly related to deforestation, extraction of natural products, hunting and exploring (Pessoa & Barreto, 1948; Castro, 1986). Age (young adults), sex (male), and low socioeconomic status are factors closely related with these occupations in the jungle areas of Brazil, Peru and Mexico (Gomes et al 1992, Bartolini, 1988; Andrade-Narvaez et al., 1992). The location of homes close to the forest has also been related to increased risk of disease (Castro, 1986; Netto et al., 1986). All of these risks have been detected on the basis of statistical association, but are not representative (in terms of sample size) or adjusted for multifactorial determinants; however, the results have provided a hypothesis for analytic studies. As a disease very closely associated with poverty, leishmaniasis usually affects the poorest people of the poorest countries; 72 of 88 countries affected are developing, and 13 of them are among the least developed. It is here, where more than 80% of the population earns less than US$ 2 per day (Davies et al., 2003), that the majority of cases are reported every year (Desjeux, 2001).

Most properly designed assessments of risk factors were published after 1990. Risk factors associated with the transmission of Leishmania infections have been demonstrated in Peru (Llanos-Cuentas, 1993; Davies et al., 1997), Colombia (Weigle et al., 1993), Costa Rica (Rojas et al., 1988), and Argentina (Yadon et al., 2003; Sosa-Estani et al., 2001), among other countries; they vary in importance and nature according to region, epidemiological and ecological characteristics, and pattern of transmission. The risk factors are quite different if the transmission is in dwellings (indoors or peridomestic) or outside the home (in forests or rural places). In addition, there is often more than one pattern of transmission in the same region. For instance, in the Andean regions where indoor transmission has been classically recognized, peridomestic and rural transmission occur at the same time. In some endemic areas, transmission around the home causes about 80% of cases; in other Andean places, where transmission occurs in rural places (Llanos-Cuentas & Davies C., 1992), a single Leishmania species (L. peruviana) may cause more than 90% of the CL cases. A similar situation occurs when the main transmission pattern is in the forest; however domestic transmission is always more frequent (Weigle et al., 1993).

The studies about risk factors suggest that ecologic and climatic characteristics, and human, vector and reservoir behaviours, together influence the transmission pattern, vector species, abundance of vectors, rate of infection of vectors and reservoirs, and incidence and prevalence of human infection. The frequency of the disease, its severity and rate of cure are more dependent on the host-parasite relationship and the antiparasitic action of the drugs used.

Despite the fact that risk factors have been determined in several countries, they have been used very little. Only in one study (Llanos-Cuentes, 1993) in Peru was estimation of the population attributable risk (PAR) for an individual subset of factors simultaneously adjusted for the risk attributable to the remaining factors in the model. A group of risk factors in central West Peru (region 1) was significantly associated with transmission inside houses because the suggested intervention was to use a single measure (spraying insecticide indoors). The combined PAR for this group was 0.792, which implies that removal of this group of factors with a specific intervention would lead to a 79.2% reduction in CL incidence in the region. In order to evaluate the impact of the same intervention measure in North-West Peru (region 2), a group of risk factors related to indoors transmission was selected and the best PAR was found to be only 5.1. For five years, both regions were systematically studied (spraying lambda-cyhalothrin in the home and peridomestic area every six months for two years, and measuring the incidence indoors transmission was selected and the best PAR was found to be only 5.1. For five years, both regions were systematically studied (spraying lambda-cyhalothrin in the home and peridomestic area every six months for two years, and measuring the incidence impact.

The studies on risk factors have taught us several lessons:

- Leishmaniasis has a dynamic epidemiology; factors can change under the influence of climate, ecology and behaviour of humans, vectors and reservoirs.
- We cannot apply the same intervention strategy in all places, even when the infection is caused by a unique species.
- There are limitations of the control strategy used in the majority of countries. The classical endpoints of control programmes are control of the disease (early diagnosis and treatment) and control of the vector(s) or reservoir(s), but in fact the primary endpoints are climate, ecology and human behaviour.

In future, leishmaniasis control programmes in developing countries should be focused on reduc-
tion of poverty and development of the health rights of the people in order to achieve sustainable success through control activities. So health and economic improvement must be critical issues in the national policy. The community should be adequately educated, in a long-term programme, about the strategies. Thus, a combined risk assessment and ecosystem approach is a new alternative based on the interrelationship between scientific knowledge, participation of the communities living in endemic areas, and economic support by the government.

References


6.2 CHALLENGES AND OPPORTUNITIES IN RESEARCH AND CONTROL

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These notes reflect personal opinion. They are deliberately controversial, and are intended rather to stimulate discussion than to determine policy. In my opinion, there has been a tendency over many years for TDR to follow what is fashionable and technologically advanced. I believe this has not only diverted TDR funds away from the goals of disease control, but has influenced other funders to do the same. TDR has a particularly important responsibility in that it serves as a trendsetter every bit as much as a sponsor of research. What follows are a few personal opinions highlighting areas where I believe we have tended to lose sight of our real objectives.

In the words of Burl Ives, let us try to ‘watch the doughnut, not the hole’.

TAXONOMY: DETECTION, DESCRIPTION, CLASSIFICATION AND IDENTIFICATION OF THE PARASITES

Taxonomy is at the very heart of almost any aspect of biology. In recent years, several happenings have affected the problem of taxonomy of Leishmania.

The advent of polymerase chain reaction (PCR) methods for amplifying DNA has greatly facilitated the detection of parasite DNA, thereby allowing detection of infection in candidate reservoir hosts.

Several studies, particularly in Sudan, have shown the standard battery of isoenzymes to be inadequate for the classification of L. donovani strains.

Microsatellite batteries promise new and more easily standardized protocols for describing series of strains.

In any taxonomic study, collection of specimens is the first objective. Long series of specimens (stocks in our instance) are required from as many populations as possible, in order to assess the variation within each population. Each stock must then be described by a range of descriptors, known as taxonomic characters. For any automated interpretation of these, there should be at least 30 characters, and the state of each should be described for each stock. With this information, operational taxonomic units can be nominated, and a classification can be compiled. Only then can limits between strains, subspecies and species be proposed, on largely subjective grounds.

Only when a reliable classification has been compiled does identification become meaningful.

Isoenzymes provided the first, and for a long time apparently ideal, set of valid, comparable taxonomic characters, and allowed the classification that largely stands today. However, several shortcomings have become apparent in the isoenzyme system, and additional methods are required, especially for certain difficult groups of strains.

When a classification has become established, marker strains of species, and marker stocks of strains, can be established. Only then may it be acceptable to reduce the number of taxonomic characters to be used for identification. Such simplification is perfectly acceptable for quantitative studies, where the range of strains has been well established. However, for stocks from new areas or from unfamiliar hosts, a full standardized description is still required.

Whether or not it is still necessary to refine the existing classification for the purposes of TDR is debatable. I suggest that there have been several instances, notably in the reports of visceralizing L. tropica, and in the apparent existence of three ‘species’ in a single Sudanese epidemic area, where the current system has been shown to be inadequate. Further, there are several instances where vectors and reservoir hosts still need to be identified. Better methods are needed in these areas.

Monoclonal antibodies, whether singly or in batteries, and so-called specific DNA probes may provide excellent tools for identification of stocks for quantitative purposes, but can be very misleading if they are over-interpreted. They are far from adequate for the ‘identification’ of new, aberrant or surprising results. Their use should be limited to the identification of stocks belonging to strains that contributed to the classification being used.

EPIDEMIOLOGY

Methods using remotely sensed information, analysed using global positioning systems (GPS) software, now allow much more detailed description of
the habitat requirements of each ‘nosodemiological unit’, and the development of detailed risk maps.

Risk maps are particularly needed in control of diseases like the leishmaniases: outbreaks are frequently mobile, and the prepatent periods are often so long that, by the time the infection is recognized in a place, very many people may have become infected. Ideally control intervention must include not only areas where the infection is known, but also peripheral areas which are suitable for transmission, where the infection may have recently been introduced but has not yet appeared.

VACCINE DEVELOPMENT

While the idea of a vaccine is all very well, we should consider whether or not it would be desirable to actually use any vaccine, and whether it might even be counterproductive to do so.

In Nepal we have estimated that a vaccine programme would need to cover some 6 million people initially, and 240 000 annually thereafter for several years at least. The number of people requiring treatment is unlikely ever to exceed 6000 annually. We estimated that, in Nepal, the unit cost of vaccination with a perfect vaccine would need to be at the very least 90 times less than that of treatment in order to be economically competitive. In the absence of any reliable model, there is no way of knowing whether a vaccine programme or an enhanced treatment programme would be more effective in controlling the disease, but an enhanced treatment programme using active case detection should have numerous beneficial side effects.

On balance, it may well be that an enhanced treatment programme would be both more cost effective and more beneficial than any vaccination programme.

I suggest that we should consider abandoning support for vaccine development, and concentrate research on:
• how to detect early cases
• how to distinguish cases who should be treated from those who will self-cure, and
• how to deliver effective treatment to every person who needs it.

This consideration is particularly appropriate for kala azar, which is relatively uncommon even in epidemics, and can be cured without lasting disfigurement, and for which the idea of an economically viable vaccine is probably ‘pie in the sky’.

DIAGNOSTIC TESTS

Many biologists justify their studies on genomics, or on the immunology of the domestic mouse, under the umbrella of ‘developing a diagnostic test’ (or more commonly but even less realistically, ‘developing a vaccine’). Usually this involves a relatively banal, stamp-collecting analysis of some antigen, justified because ‘it might lead to a diagnostic test’ (in fact, the most it will usually do is teach a student some fashionable techniques).

Almost always, once a ‘test’ has been ‘developed’ (usually in a PhD-with-no-Ph project), it is promptly set aside in favour of the next fashionable study. Such studies should be justified in terms of their potential contribution to training, or to basic science (not much, usually), and should not be funded under the banner of tropical diseases research.

As I see it, any diagnostic test may serve one or more of at least four main functions; each function requires a test with particular specifications. Table 1 below compares some of the specifications desirable of tests in the main diagnostic contexts.

Only rarely is the purpose of diagnosis precisely described, and only exceptionally is a project specifically aimed at a clearly defined goal. Diagnostics research to be funded by TDR should be clearly identified within one or more categories of test, and the potential advantages over existing tests should be clearly identified.

CONCLUSIONS

After all the years of TDR, now is a good time to take stock of achievements, not in terms of scientific, personal or institutional interest, but in terms of relief of suffering.

The greatly improved classification of the parasites has allowed meaningful identification and, thereby, enormous improvement of our understanding of the structure of both zoonotic and anthropoanotic foci. Serious gaps still exist in this vital area.

First the direct agglutination test (DAT), then the dipstick rK39 and, dare we hope, the urine antigen test, materially advance diagnosis at village clinic and treatment centre level. Rapid, reliable diagnosis must be the basis for intervention relying on active case detection and treatment.

Developments in treatment are limited: excellent new drug formulations or combinations have not radically reduced the cost, nor the duration of
treatment. Oral miltefosine promises to be advantageous, but has serious limitations.

Vaccination remains pie in the sky.

We know little more of sandfly ecology than we did at the last expert committee, and that was not much. Sandfly ecology remains one of the most important areas where research is required.

Our work in Tunisia developed guidelines for prediction of zoonotic cutaneous leishmaniasis (ZCL) outbreaks: these need to be refined and could then easily be implemented. As ever, it is the link between the ministry and the laboratory that needs to be reinforced.

As far as kala azar in the Indian subcontinent is concerned, current technology should be quite adequate for the disease to be eliminated to the extent that there are very few cases and no deaths. It is the implementation of such technology that is wanting. The same is probably true of infantile visceral leishmaniasis. For both of these, the emphasis of research should be on the socio-politico-economic aspects, advocacy, and delivery of education to the politicians, medical profession and villagers.

Table 1. Some specifications for clinical and epidemiological diagnostic methods

<table>
<thead>
<tr>
<th>Aim</th>
<th>Objective</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Expense</th>
<th>Reference material</th>
<th>Direct/indirect</th>
<th>Shelf life</th>
<th>Technical level of operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indicates: To treat or not to treat</td>
<td>Must identify active infection</td>
<td>Screening test (optional) must be highly sensitive</td>
<td>Specificity important at species level</td>
<td>Expense not the major issue</td>
<td>Positive and negative controls required</td>
<td>Indirect/direct tests OK</td>
<td>Important, especially in acute or rare diseases</td>
<td>Clinician/clinical technologist, should be basic level</td>
</tr>
<tr>
<td>Indicates: To refer for confirmation or not</td>
<td>Must detect all active infections</td>
<td>Screening test must be highly sensitive</td>
<td>(Refer positives to PCD methods)</td>
<td>Must be very cheap</td>
<td>Reference material of lesser importance – refer positives to specific test</td>
<td>Indirect/direct tests OK</td>
<td>Unimportant – if used in special projects</td>
<td>Technologist, may require high level</td>
</tr>
<tr>
<td>Indicates: Density and dynamics of agent in host or vector population</td>
<td>Should distinguish infecteds, susceptibles and immunes</td>
<td>Single test desirable; sensitivity must be known</td>
<td>Specificity must be known</td>
<td>Must be very cheap</td>
<td>Reference material of lesser importance – test must be well calibrated</td>
<td>Indirect/direct tests OK</td>
<td>Unimportant – usually limited to special projects</td>
<td>Technologist, may require high level</td>
</tr>
<tr>
<td>Indicates: Presence of agent, by geography, host and vector</td>
<td>Must identify active infection</td>
<td>Sensitivity not important</td>
<td>Specificity essential, to strain level</td>
<td>Complex, expensive procedures acceptable</td>
<td>Reference collection material vital</td>
<td>Direct test essential (parasite or DNA)</td>
<td>Research scientist: may involve hi-tech for identification of agent</td>
<td></td>
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Annex 7

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7.1 DRUG RESISTANCE IN LEISHMANIASIS

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SUMMARY

The efficacy of drugs used in the treatment of visceral and cutaneous leishmaniasis is influenced by both host and parasite factors. The latter includes both an intrinsic variation in the sensitivity of Leishmania species as well as acquired drug resistance due to selection. Intrinsic variation in sensitivity has been described to pentavalent antimonials, paromomycin, miltefosine, azoles and other drugs that have reached clinical trials. Acquired resistance to anti-leishmanial drugs has been reported from laboratory studies over several decades but it is only recently that this has been shown in Leishmania donovani visceral leishmaniasis clinical resistance to antimonials. The development of resistance to antimonials and the potential for resistance to other drugs has several implications. It makes the search for new drugs more urgent, it focuses attention on our lack of knowledge about the molecular and biochemical mechanisms of action of antimonial drugs on Leishmania, it places emphasis on strategies and policies for use of anti-leishmanial drugs in endemic areas, and it brings into focus the need to consider drug combinations in anti-leishmanial therapy. There is a need to develop systems for the monitoring and surveillance of resistance. However, for antimonials and most drugs this is problematic due to the absence of molecular markers and a reliance on the amastigote-macrophage culture assay to adequately correlate clinical response with parasite phenotype.

INTRODUCTION

Leishmaniasis is a disease complex caused by species of haemoflagellate protozoa parasites belonging to the genus Leishmania. There are an estimated 12 million humans infected, with an incidence of 0.5 million cases of the visceral form of the disease and 1.5 to 2.0 million cases of the cutaneous form of the disease. The numbers are probably underestimated as leishmaniasis is a reportable disease in only 40 of the 88 countries worldwide (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm) where it is known to be present. In addition to the two major clinical forms of the disease, visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), there are other cutaneous manifestations including mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), recidivans leishmaniasis (LR) and post kala azar dermal leishmaniasis (PKDL). The current situation for the chemotherapy of leishmaniasis is more promising than it has been for several years with both new drugs and new formulations of old drugs either recently approved or on clinical trial (Croft and Coombs, 2003; Croft and Yardley, 2002; Murray, 2001). In the past decade, four new potential therapies for VL have been introduced: a parenteral formulation of aminosidine (paromomycin) (Thakur et al., 2000), amphotericin B liposomes (Berman et al., 1998; Meyerhoff, 1999), and the orally active drugs miltefosine (Sundar et al., 2002) and sitamaquine (WR6026) (Sherwood et al., 1994; Dietze et al., 2001). Treatment of CL has been improved by various topical formulations of paromomycin (El-On et al., 1992; Asilian et al., 2003; Soto et al., 2002; reviewed Garnier and Croft, 2002) and could also benefit from oral miltefosine (Soto et al., 2001). Several other drugs, in particular the antifungal azoles itraconazole, ketoconazole and fluconazole, have been on limited clinical trials, often with equivocal results.

Drug treatment is complicated by the variation in sensitivity of Leishmania species, the different disease manifestations, the variation in host response, and the absence of baseline data from controlled clinical trials of many old drugs for CL. The aims of this review are to dissect the problems of variation in drug sensitivity and acquired drug resistance, to evaluate their importance in anti-leishmanial chemotherapy and, finally, to consider implications for drug use, research, surveillance, and public health.

CONFOUNDING ISSUES – IMMUNE STATUS AND PHARMACOKINETICS

Firstly, it is important to briefly acknowledge that host factors affect drug efficacy and must be considered in interpretation of data. The immune status of leishmaniasis patients has long been known to effect drug activity. This has proved to be of particular importance in relation to pentavalent antimonial treatment of DCL (Ercoli, 1966) and HIV/VL co-infections (Alvar et al., 1997; Berhe et al., 1999) where there is an absence of specific T cell-mediated immune response and mutual exacerbation of infection. The basis of this lack of activity of pentavalent antimonials has been explored in immunodeficient mouse models where the effects are probably due to deficiencies of both Th1 cell-mediated and macrophage responses (Murray et al., 1989; Murray...
and Delph-Etienne, 2000). Experimental models have also shown that the anti-leishmanial activities of amphotericin B and miltefosine are T cell independent, whereas pentamidine is T cell dependent (Murray et al., 1993; Murray & Delph-Etienne, 2000; Escobar et al., 2001).

As the sites of infection in leishmaniasis are normally in either the visceral organs or the skin, pharmacokinetic properties can also affect drug efficacy. These properties could influence the activity of sitamaquine, an 8-aminoquinoline, a class of drugs that is well distributed to the liver (Carson, 1984), or itraconazole (a triazole), that is well distributed to the skin (Leyden, 1998). Other pharmacokinetic properties, metabolism and excretion, must also be considered. In a study on the treatment of CL in Saudi Arabia, patients showed marked variation in response to Pentostam, but no differences were observed in the sensitivity of Leishmania major isolates to this drug from patients in the amastigote–macrophage model (Al-Jaser, 1995). However, significant differences were observed between patients in the elimination rate of antimonials, and area under the curve (AUC) analysis suggested that differences in the length of exposure to antimony could influence response in CL treatment (Al Jaser et al., 1995).

**DRUG RESISTANCE – THE PROBLEM**

**Drug sensitivity and Leishmania species**

There are over seventeen species of Leishmania known to be infective to humans and they have been characterized by biochemical and molecular differences that provide the basis for phylogenetic analysis and several methods of diagnosis (Cupolillo et al., 2000). These biochemical and molecular differences between species are reflected in variation of the intrinsic sensitivity of Leishmania species to a range of drugs from different chemical classes. Examples of intrinsic variation have been defined in a number of laboratory studies, but interpretation of these studies is important as different assay conditions lead to several-fold differences in activity values (Croft and Brun, 2003).

**Antimonials**

The variation in clinical response to the pentavalent antimonials sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) has been a persistent problem in the treatment of leishmaniasis over the past 50 years. There is an intrinsic difference in the sensitivity of species to these antimonials. In studies using the amastigote-macrophage model, L. donovani and L. braziliensis were 3-5 fold more sensitive to sodium stibogluconate than L. major, L. tropica and L. mexicana (Allen and Neal, 1989; Neal et al., 1995). Other studies by Berman et al. (1982), using another amastigote-macrophage model, and Grogl et al (1992), using promastigote cultures, demonstrated wide variation in the sensitivity of isolates from cutaneous leishmaniasis cases to pentavalent antimonials, and suggested that there was a correlation between sub-curative treatment and decreased clinical drug sensitivity. This has been confirmed in one of the few controlled clinical trials that compared the cure rate to antimonials of CL caused by different species. Pentostam produced a significantly higher cure rate for L. braziliensis lesions than for L. mexicana lesions (Navin et al., 1992).

**Paromomycin (aminosidine)**

This aminoglycoside antibiotic has been used for the treatment of both VL, in a parenteral formulation, and CL, in both topical and parenteral formulations. In experimental models and in clinical treatment, lesions caused by L. major that were treated with paromomycin ointment resolved faster and more completely than lesions caused by L. amazonensis and L. panamensis (El-On & Hamburger, 1987). These observations have been supported by an in vitro study of the sensitivity of amastigotes, where L. major and L. tropica (ED50 values in the range of 1–5 µM) were more sensitive than L. braziliensis (ED50 < 12µM) and L. mexicana (ED50 39µM), while L. donovani showed intermediate sensitivity (ED50 6–18 µM except DD8 strain which had an ED50 > 150µM) (Neal et al., 1995).

**Amphotericin B**

This polyene antibiotic has selective activity against fungi, as well as Leishmania and Trypanosoma cruzi, through a higher affinity for the predominant microbial sterol ergosterol than for host cell cholesterol. Differences in species sensitivity might be expected due to variation in the type and quantity of sterols in membranes of different species (Beach et al., 1988), and have been characterized in relation to ergosterol biosynthesis in fungi (Young et al., 2003). These observations were extended in a recent in vitro study on amastigotes of six species in macrophages, where L. mexicana was less sensitive to this antibiotic (Escobar et al., 2002). The higher efficacy of liposomal amphotericin B (AmBisome) against L. donovani than L. infantum/L. chagasi infections (Berman et al., 1998) is probably unrelated to species sensitivity, and more affected by parasite load, pharmacology and pathology.
Azoles

The biosynthetic pathway of ergosterol, the major sterol in fungi as well as *Leishmania* spp. and *T. cruzi*, is a target for the most important antifungal drugs. There has been an interest in two classes of these drugs as anti-leishmanial agents, the allylamines (for example, terbinafine) that inhibit squalene epoxidase, and the azoles (for example, ketoconazole and itraconazole) that inhibit C14α-demethylase. A number of clinical studies have suggested that these sterol biosynthesis inhibitors are more effective against *L. major* and *L. mexicana* infections than against *L. donovani* or *L. braziliensis* infections. One placebo controlled trial on the treatment of CL showed that *L. mexicana* infections were more responsive than *L. braziliensis* infections to ketoconazole (Navin et al., 1992). The results from in vitro studies that have investigated the intrinsic differences in sensitivity of *Leishmania* species to sterol biosynthesis inhibitors support this clinical observation. In a comparative study on the sensitivity of promastigotes to ketoconazole, *L. donovani*, *L. braziliensis* and *L. amazonensis* were found to be more sensitive than *L. aethiopica*, *L. major*, *L. tropica* and *L. mexicana* (Beach et al., 1988). However, these results contrast with those of Rangel et al. (1996), who found that *L. braziliensis* was relatively insensitive to ketoconazole and the bis-triazole D0870 whereas *L. mexicana* was sensitive to ketoconazole. Both sets of results contrast with an earlier study using an amastigote-macrophage model, which showed that *L. donovani* was more sensitive to ketoconazole than *L. mexicana* or *L. major* (Berman, 1982); in this case the ability of amastigotes to salvage sterols from host cell macrophages might play a part (Roberts et al., 2003).

Nucleoside analogues

Wide variations in the sensitivity of promastigotes of different species to the pyrazolopyrimidines allo-purinol and allo-purinol riboside have been reported, suggesting differences in the affinity of enzymes of the purine salvage pathway (Avila and Casanova, 1982; Nelson et al., 1979).

Miltefosine (hexadecylphosphocholine) and edelfosine (ET-18-OCH$_3$)

Variation in the sensitivities of promastigote and amastigote stages of *L. donovani*, *L. major*, *L. tropica*, *L. aethiopica*, *L. mexicana* and *L. panamensis* were shown in vitro to these phospholipid drugs (Escobar et al., 2002). In all assays, *L. donovani* was the most sensitive species, with ED$_{50}$ values in the range of 0.12 to 1.32 µM against promastigotes and 1.2 to 4.6 µM against amastigotes. *L. major* was the least sensitive species in the majority of assays, with ED$_{50}$ values for miltefosine in the range of 4.8 to 13.1 µM against promastigotes and for HPC and ET-18-OCH$_3$ in the range of 7.5 to 37.1 µM against amastigotes.

Sitamaquine

There have been no direct comparative studies. Earlier studies reported similar ED$_{50}$ values for two species, 2.6 µM against *L. tropica* (Berman and Lee, 1983) and 1.5 µM against *L. donovani* (Neal and Croft, 1984).

Acquired drug resistance

The selection of drug resistant pathogens is a major and well known threat to the treatment of bacterial
infections as well as malaria. However, although the selection of resistant Leishmania has long been a part of laboratory studies, it is only in the past decade that acquired resistance has become a clinical threat, and this is mainly restricted to pentavalent antimonial drugs in India.

**Antimonials**

Studies over the past decade have demonstrated an ever decreasing response rate of VL cases to antimonial treatment in Bihar, India (Figure 1) and that, in this major focus of VL, over 60% of cases are treatment failures to pentavalent antimonials (Sundar, 2001). This has had a major impact on the treatment of visceral leishmaniasis. It is important to note that the focus of resistance is in districts of Bihar north of the Ganges, in comparison to Bihar districts south of the Ganges and neighbouring Uttar Pradesh where patients remain responsive to antimonial treatment (see map in Sundar, 2001).


So is this failure of response due to acquired resistance? It must be assumed that the problems outlined by Sundar are also to be found in districts where antimonials are still relatively effective. In a study to determine whether acquired resistance was present in Bihar, L. donovani isolates were taken from responders and non-responders (Lira et al., 1999). In vitro, amastigote–macrophage isolates from patients who did respond to Pentostam were three fold more sensitive (ED50 values around 2.5 µg Sb/ml) compared to isolates from patients who did not respond to Pentostam (ED50 values around 7.5 µg Sb/ml). There was no difference in the sensitivity of isolates when the promastigote assay was used (Lira et al., 1999). The significant difference in amastigote sensitivity and the correlation supports acquired resistance. However, the sample size was small (15 non-responders, 9 responders), and a three-fold difference in sensitivity can be seen between experiments in this model (Croft and Brun, 2003). As already mentioned, there are strains in circulation that are insensitive to antimonials. An earlier report on VL isolates from Sudan also showed that clinical response to Pentostam was reflected in the amastigote-macrophage model (but not in promastigotes) in an area where there was still a good response to antimonials (Ibrahim et al., 1994). More biological evidence is required to support the temporal and spatial parameters of the Bihar phenomenon. Other biological data do support the idea of acquired resistance. The potential for selection of antimonial resistance in L. infantum has been reported from immunodeficient and immunocompetent VL patients in France (Faraut-Gambarelli et al., 1997). Isolates from 14 patients were made prior to and after Glucantime treatment, and their sensitivity was measured in an amastigote–macrophage assay. Thirteen of the 14 isolates were less sensitive to the antimonial after treatment. Similar decreased sensitivity was observed in L. infantum isolates taken from dogs before and after Glucantime treatment (Gramiccia et al., 1992). In the laboratory, L. donovani resistance to antimonials is easily generated, for example in promastigotes (Grogl et al., 1989), a rodent model (see Berman et al., 1982), and more recently in axenic amastigote cultures of L. donovani and L. infantum (Ephros et al., 1997; Sereno and Lemesre, 1997).

So, if there is acquired resistance, do we have a handle on the mechanisms? After 60 years of use, the anti-leishmanial mechanism of action of pentavalent antimonials is still not clearly defined and this makes a full description of mechanism of resistance impossible. However, recent studies have pointed to particular biochemical events that are involved in antimonial sensitivity (Figure 2):

- The lack of sensitivity of promastigotes and the sensitivity of amastigotes to pentavalent antimonials depends upon transformation of pentavalent compound to trivalent compound in the latter but not the former stage (Shaked-Mishan et al., 2001; Santos Ferriera et al., 2002). The question is whether “resistant” amastigotes are also deficient in their ability to reduce SbV to SbIII? A recent report started to make some connections: in vivo L. panamensis amastigotes derived from an SbIII resistant promastigote line were more resistant to SbV treatment than wild type (Travi et al., 2003). The accumulation of SbV and SbIII into promastigotes and amastigotes has been shown to be by different transport systems (Brochu et al., 2003), and though Sb accumulation was lower in resistant than sensitive forms, levels of accumulation could not be correlated to sensitivity in wild type cells.

- Whatever, the role of the parasite is unlikely to be an absolute factor as thiols of host cell macrophages also reduce SbV to SbIII (Frezard et al., 2001; Santos Ferriera et al., 2002).

- Parasite thiols (trypanothione, glutathione) are considered by many to have a critical role in the process of detoxication of heavy metals in trypanosomatids. Studies on trivalent antimony (and arsenic) resistant Leishmania parasites have
shown increased levels of thiols (trypanothione, glucathione, ovothiol). In these lines, this has been related to amplification of enzymes involved in the synthesis of glutathione (γ-glutamylcysteine synthetase, gsh1) and polyamines (ornithine decarboxylase), the two precursor molecules of trypanothione, in some studies (Haimer et al., 2000; Mukhopadhyay et al., 1996) but not others (Arana et al., 1998).

- The next part of detoxification is the conjugation of antimony with a thiol. Glutathione-S-transferase (GST) could mediate this activity. Increased GST expression and levels have recently been reported in antimony resistant isolates with concomitant increased levels of spermidine and glutathione (Chatterjee et al., 2003). A trypanothione-S-transferase, if present, could play a role here as well.

- The efflux of Sb is the final step. Sb-thiol conjugates are amplified in some cases (Arana et al., 1998; Haimer et al., 2000; Ouellette et al., 1998), effluxed from Leishmania by PGPA-like ABC transporters (Roberts et al., 1995), or sequestered into a vacuole (Legare et al., 2001).

Two notes of caution: many of the studies on this complex process have been carried out in vitro on the promastigote stage which, as already described, has many differences to the relevant amastigote stage (ibid); and some studies were performed on L. tarentolae, a distant relative of L. donovani which has, for example, no transporter for miltefosine (Perez-Victoria et al., 2003b). In field isolates, no amplification of genes seen in laboratory studies was observed; rather an amplification on chromosome 9 was related to protein phosphorylation (Singh, Singh and Sundar, 2003).

\[\text{Sb}^\text{III} - \text{thiol conjugate} \rightarrow \text{GSH} \rightarrow \text{PGPA transporter} \rightarrow \text{vesicle} \rightarrow \text{ODC} \rightarrow \text{GST} \rightarrow \text{Sb}^\text{III} \rightarrow \text{Sb}^\text{V} \]

**Figure 2. Proposed Sb metabolism in Leishmania**

**Amphotericin B**

Although this antibiotic has been widely used in the treatment of mycoses for over 30 years, resistance in fungal isolates has been reported only rarely (Georgopapadakou, 1998). However, resistance is possible, and a resistant L. donovani clone of promastigotes was selected through a step-wise increase in amphotericin B concentration in culture. Resistant promastigotes showed a significant change in the plasmamembrane sterol profile by gas chromatography/mass spectrometry, ergosterol being replaced by a precursor cholesta-5, 7, 24-trien-3β-ol (Mbongo et al., 1998). This is probably due to an effect on C24-transmethylation due to changes in S-adenosyl-L-methionine-C24-Δ-sterol-methyltransferase (SCMT) (Loiseau et al., 2002), the same enzyme that confers resistance to some Candida (Young et al., 2003). There have been two small inconclusive studies on the emergence of amphotericin B resistance in L. infantum/HIV infected cases in France. One study failed to find a change in sensitivity in isolates taken before and after treatment of one patient (Durand et al., 1998). In contrast, a decrease in sensitivity was observed in isolates taken over several relapses from another patient (Di Giorgio et al., 1999). There has been increased use of amphotericin B for visceral leishmaniasis, both in the deoxycholate (Thakur et al., 1999; Sundar et al., 2002) and lipid formulations (Berman et al., 1998; Sundar et al., 2003) following failure of antimodal treatment. Although amphotericin B resistant L. donovani promastigotes have been selected by increasing drug pressure (Mbongo et al., 1998), this has yet to be demonstrated in amastigotes or under field conditions.

**Paromomycin**

This aminoglycoside is in phase III trials as a parental formulation for VL and in topical formulations for CL (two commercial products). The mechanisms of resistance in bacteria to this class of antibiotic are well characterized; most commonly the drug is metabolized to an inactive form by acetylases and phosphorylases. In studies on selected populations of promastigotes, resistance was related to decreased drug uptake in L. donovani (Maarouf et al., 1998), but not to enzymatic modification or to any mutation of the small sub-unit ribosomal gene in L. tropica (Fong et al., 1994). The mechanisms of resistance to paromomycin in Leishmania have not been sufficiently defined.

There is potential for the development of clinical resistance. Following a 60-day parenteral course for L. aethiopica cases, and using an amastigote–macrophage assay, isolates taken from relapse patients were 3-5 fold less sensitive to the drug after treatment
than isolates taken before treatment (Teklemariam et al., 1994). Monitoring of resistance could be of importance if the parenteral formulation is introduced as a first line treatment for L. donovani in an anthroponotic focus.

**Miltefosine**

Promastigote lines of L. donovani resistant to hexadecylphosphocholine were generated in the laboratory. Resistance was stable after withdrawal of drug pressure, but the lines showed little cross-resistance to standard anti-leishmanial drugs (Seifert et al., 2003). The mechanism of resistance in the 40 \( \mu \text{M} \) miltefosine resistant line has been determined as due to defective uptake of miltefosine (Perez-Victoria et al., 2003a) through point mutations on a plasmamembrane aminophospholipid translocase (Perez-Victoria et al., 2003b). The potential relevance of these observations needs to be extended to miltefosine-resistant amastigotes before clinical implications can be properly considered. In addition, it has been shown that multidrug resistant L. tropica lines that overexpress a P-glycoprotein are less sensitive to miltefosine (Perez-Victoria et al., 2001).

**Sitamaquine**

L. donovani promastigote lines resistant to sitamaquine have been selected in the laboratory (Philippe Loiseau, pers. comm.), but no further information is available.

**Pentamidine**

Pentamidine has been used as a second line treatment for VL, CL and DCL, but its anti-leishmanial mechanism of action, after 40 years of use, is still not defined (see Bray et al., 2003). It was shown that pentamidine resistant clones of L. donovani and L. amazonensis promastigotes have reduced uptake and increased efflux of the drug due to specific transporters (Bray et al., 2003; Coelho, Beverley & Cotrim, 2003). A recent review of resistance to diamidines in protozoa also noted the importance of drug accumulation into the Leishmania mitochondrion (Bray et al., 2003). The limited use of pentamidine, often in a zoonotic setting, suggests that resistance should not be a problem.

**WHAT TO DO ABOUT THE PROBLEM?**

In a 2001 review, Bryceson stated “At the moment, there seems to be no policy at an international or national level to prevent the emergence of parasite resistance to anti-leishmanial drugs”. Given the problems discussed above, and the tools available, perhaps we have to address the measures and policy that should be put into place.

Two important considerations in analysing the seriousness of the drug resistance problem are (a) the ease with which resistant individual microbes can be selected for by a particular drug, and (b) potential spread of resistance in a population and therefore the public health importance. Firstly, the spread of drug resistant genotypes through a population of microorganisms is primarily governed by certain measurable parameters: (i) the volume of drug used, (ii) the probability that a drug-sensitive infection becomes resistant upon infection, (iii) the duration of infection in individuals, (iv) the fitness costs (division rate, transmissibility) for the pathogen incurred by being resistant in the absence of drugs, and (v) the degree to which compensatory mechanisms develop that offset these fitness costs (Bjorkman et al., 2000; Davison, Low & Woolhouse, 2000; Levin, Perrot & Walker, 2000). In zoonotic diseases, like most cutaneous leishmaniasis and most L. infantum/ L. chagasi visceral leishmaniasis, the parasite is primarily an infection of a feral or domestic mammalian host and only occasionally humans. The volume of drug used in relation to the time that a parasite population is exposed to a drug is insignificant for most forms of leishmaniasis unless the mammalian host is also treated. This is a consideration when control methods for canine leishmaniasis are implemented, where extensive treatment of the domestic canine host has been shown to lead to changes in sensitivity of isolates (Gramiccia, Gradoni & Orsini, 1992; Gradoni, Gramiccia & Scalone, 2003). Effects of drug resistance on the fitness (and virulence), for example on the abilities to transform, to establish an infection in the sandfly vector, to outgrow a non-resistant wildtype, and \( R_0 \) of Leishmania, have not been estimated. Current knowledge of the epidemiology and transmission of leishmaniasis suggests that the spread of acquired drug resistance is not a factor to be considered in cutaneous leishmaniasis, except maybe in anthroponotic foci of L. tropica, but that it is a factor that requires consideration in L. infantum leishmaniasis, where there is needle transmission (Alvar et al., 1997). However, it is clearly a factor of major importance in anthroponotic disease foci such as L. donovani in Bihar State, India (Sundar, 2001). This does not preclude a number of studies that describe the development of resistance in parasites normally associated with zoonotic infections in animals (Gramiccia Gradoni & Orsini, 1992) or in humans during long courses of treatment, especially in immunocompromised patients (Faraut-Gambarelli et al.,1997); rather, these studies must be seen in the context of the wider population. Also, it is not ignoring observations that indicate that
within zoonotic populations of leishmaniasis there are populations that are highly drug insensitive (e.g. Grogl et al., 1992). These populations probably have a highly stable “resistance” phenotype (and genotype) and are transmitted from host to host.

So what strategies are available to combat the problems of drug resistance?

**Monitoring drug resistance**

In all cases we require better methods to monitor drug resistance through either (i) phenotypic sensitivity of parasite isolates, or (ii) molecular changes that indicate alterations in either the drug target or mechanisms that alter the intra-parasite level of active drug.

There are problems with both approaches:

(i) The determination of drug sensitivity of clinical isolates is open to the criticism that pathogen adaptation from host to culture medium immediately selects for a sub-population of pathogens best suited for growth in that medium. The drug sensitivity of parasites must therefore be tested as soon as possible after isolation from the patient using carefully defined protocols. Although promastigote assays are easiest and quickest, this assay is not predictive for pentavalent antimonials, and possibly not for other anti-leishmanials, for example paromomycin, pentamidine and miltefosine. The amastigote-macrophage assay is currently the only model able to correlate clinical response to the sensitivity of the isolate, as demonstrated in relation to pentavalent antimonials (Ibrahim et al., 1994; Lira et al., 1999). Axenic amastigotes are sensitive to antimonials but adaptation of isolates is both too selective and too lengthy a process to be used in this type of assay (Ephros, Waldman & Zilberstein, 1997; Sereno and Lemesre, 1997).

(ii) The ability to develop molecular probes or polymerase chain reaction (PCR) based diagnostics to monitor the development and spread of drug resistance is severely limited by a lack of knowledge of the molecular and biochemical mechanisms of action and resistance of most anti-leishmanial drugs, especially in clinical isolates (see above). Molecular techniques using cosmid vectors have been successfully used to identify drug resistant loci in *Leishmania*, in particular those to antifolates, pentamidine and sterol biosynthesis inhibitors (Cotrim, Garrity & Beverly, 1999; Coelho, Beverley & Cotrim, 2003).

**DOTS**

The introduction of oral drugs for leishmaniasis offers advantages of improved compliance, self-administration and reduced costs. In the phase IV trial for miltefosine, a seven-day supply is issued to patients who have to return to the clinic each week for examination and re-supply. For drugs like miltefosine which have a long half-life and a propensity for selection of resistant forms, the monitoring of daily dosing and the completion of a course of treatment is essential. The directly observed treatment (DOTS) strategy for TB chemotherapy was successfully introduced in India by the Revised National TB Control Programme (RNTCP) in 1997 (see WHO Report, 2003. Global Tuberculosis Control – www.who.int/gtb/publications/globerep/index.html). The potential for use of a parallel system for the control of leishmaniasis, both for miltefosine now and maybe sitamaquine in the future, should be considered.

**Diagnostic methods**

The development of non-invasive serological diagnostic methods with high sensitivity and specificity, for example DAT, K39, and Katex (urine dipstick), is a major advance in the control of leishmaniasis (see Guerin et al., 2002). In this context, it is important to evaluate these kits for the monitoring of drug response in patients. The antigen detecting katex (Sakari, Chance & Hommel, 2002) might be of particular interest in this case.

The variation in species sensitivity has greatest clinical significance in Central and South America where the distribution of *L. mexicana*, *L. amazonensis*, *L. panamensis*, *L. braziliensis* and other members of these groups overlap. Diagnosis by microscopy is possible for trained staff, otherwise molecular tools have been developed that could be implemented.

**Combinations**

Drug combinations have proved to be an essential feature of antimicrobial treatment through design or use to: (i) increase activity through use of compounds with synergistic or additive activity, (ii) prevent the emergence of drug resistance, (iii) lower required doses, reducing chances of toxic side effects and cost, and/or (iv) increase the spectrum of activity, for example anti-leishmanial and anti-inflammatory or immunomodulator in cutaneous leishmaniasis. Previous studies on drug combinations for VL, for example allopurinol plus sodium stibogluconate (Chunge et al., 1985) and paromomycin plus sodium stibogluconate (Chunge et al., 1990; Neal et al., 1995; Thakur et al., 2000), have aimed to improve efficacy.
The use of combinations to prevent resistance has been well rehearsed for antimalarials. Resistance due to point mutations has been estimated in symptomatic individuals that harbour up to about 10^{12} parasites. If a target enzyme has a mutation rate of 10^{-7}, the chance of resistance developing to a single agent is high, but the likelihood of resistance to two compounds with different targets is very low (White and Pongtavornpinyo, 2002). Studies to identify such combinations are new for leishmaniasis; limited studies are under way to examine the interactions between miltefosine and other anti-leishmanials to identify suitable combinations (Seifert and Croft, unpublished). Bryceson (2001) has made the case for examining combinations of strong anti-leishmanials with “weak” drugs (for example, azoles); this is an approach also used in malaria treatment, for example the inclusion of clindamycin or azithromycin in combinations. Combinations should also consider both simultaneous and sequential administration.

Reversal agents

The strategy to reverse resistance has long been discussed in relation to chloroquine resistance in *Plasmodium falciparum*, and has looked interesting experimentally but not clinically. In laboratory studies, a series of sesquiterpenes have been shown to reverse drug resistance due to P-glycoproteins in a *L. tropica* clone (Perez-Victoria et al., 2002). Another study suggested a strategy of inhibition of thiol levels by co-administration of antimony with an inhibitor of glutathione synthesis (Carter et al., 2003). It is, however, unlikely that these approaches will have any clinical relevance.

New targets, new drugs

There are few better ways to avoid drug resistance than to have an adequate armoury of drugs with different targets and no cross-resistance. Although miltefosine, paromomycin and sitamaquine are in various stages of development for leishmaniasis treatment (Croft and Coombs, 2003), not all have completed the final steps for registration and all have clear limitations. There are still insufficient data as to whether the compounds fulfil all necessary criteria and a need for new drugs.

CONCLUSIONS

Variation in the efficacy of drugs in the treatment of leishmaniasis is frequently due to differences in drug sensitivity of *Leishmania* species, the immune status of the patient, or the pharmacokinetic properties of the drug. Most leishmaniasis is zoonotic, and acquired drug resistance is not an important consideration. In areas of anthroponotic visceral leishmaniasis however, especially India, acquired resistance to pentavalent antimonials has occurred and effective monitoring of drug resistance is needed. No molecular markers of resistance are available for currently used anti-leishmanial drugs. The only reliable method to monitor resistance of isolates is the technically demanding in vitro amastigote-macrophage model. New treatments for visceral leishmaniasis have been introduced and others are on clinical trial. Care needs to be taken that resistance does not develop to these drugs, and regimens of simultaneous or sequential combinations need to be considered as well as systems to monitor drug use, drug response and spread of resistance.

Acknowledgements

Thanks to all the team of the EC INCO-DEV project “Molecular tools for monitoring emergence and spread of drug resistance among natural populations of Leishmania” [ICA4-CT-2001-10076], led by Jean-Claude Dujardin, for many stimulating discussions.

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7.2 FROM GENOME TO DISCOVERY IN LEISHMANIA: RECOMMENDATIONS FOR THE DEVELOPMENT OF DRUGS, VACCINES AND DIAGNOSTICS

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INTRODUCTION

The completion of the Leishmania major genome sequence is a landmark that, for the first time, identifies all genes that encode proteins and processes that may be targets for drug development and vaccine candidates, and useful for diagnostics. However, it is not known how these pathogens utilize the information in their genome to establish infection, prevent elimination by the host, and cause disease.

Recent technological advances in experimental and in silico analyses create a promising environment for generating the knowledge needed to combat the spectrum of leishmanial diseases. These can be utilized, for example, for comparative genomics at both the gene sequence and RNA expression levels, for parasite and host cell expression profiling at the RNA (DNA microarrays) and protein (mass spectrometry) levels, and for comprehensive analyses of host immune responses to infection or experimental immunization. This review summarizes the current status and recommends future directions for improving the diagnosis, prevention and treatment of leishmaniasis using genome-wide technologies.

DIAGNOSIS OF LEISHMANIASIS

Current methods in molecular diagnosis of leishmaniasis

Several methods are used for diagnosis of leishmaniasis; these can be classified into three groups – parasitological, serological, molecular. Reliable diagnosis of leishmaniasis is made by demonstrating the parasite in biopsy samples or tissue aspirates of the spleen or bone marrow. Identification by microscopy can be either by direct examination of tissue smears or following tissue culture or animal inoculation. Direct examination works best for visceral leishmaniasis (VL) due to parasite density in the smears, with spleen aspirates being more sensitive than bone marrow or lymph node aspirates. Parasitological diagnosis of cutaneous leishmaniasis (CL) is made by demonstrating amastigotes in skin lesions using skin biopsy and culture of these specimens. Culture-based diagnosis of mucocutaneous leishmaniasis has very low sensitivity as the organisms are often scant.

Serological diagnosis is based on the presence of a specific humoral response, as in visceral leishmaniasis, or on the cell-mediated immune response, as in cutaneous and mucocutaneous leishmaniasis [1]. A wide range of serological methods, which vary in sensitivity and specificity, are available for the diagnosis of visceral leishmaniasis; these tests can be grouped into non-specific and specific. Non-specific tests have been used in the past but are rarely used today; they include: indirect haemagglutination, counter-current immunoelectrophoresis, and immunodiffusion. These are cumbersome tests and tend to have lower sensitivity and specificity; an example is the Montenegro skin test, a delayed-type hypersensitivity test. To date, no specific antigen has been identified as a standard for this test, although a few candidates are currently being studied in TDR-financed projects.

Specific serological diagnostic methods include the direct agglutination test (DAT), the immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), immunoblotting, and antigen detection [4].

The DAT is highly specific, sensitive, simple, and inexpensive; it uses whole, stained promastigotes either in suspension or in freeze-dried form. The freeze-dried form is heat stable and enables application of the DAT in the field. However, the DAT has a major disadvantage: the relatively long incubation time of 18 hours and the requirement for serial dilution of serum [5, 6]. Also, the DAT has no prognostic value, since the test may remain positive for several years. Recently, a modified version of DAT, the fast agglutination screen test (FAST), has been developed for use in endemic regions and for large-scale population screening.

The IFAT is one of the most sensitive tests available. It is based on detecting antibodies that appear in the very early stages of infection and are undetectable six to nine months after cure. If the antibodies persist in low titres, it is good indication of relapse [2, 3].
The ELISA is the most commonly used test for immunodiagnosis of leishmaniasis. The antigens used are traditionally derived from axenic promastigotes and consist of a repertoire of at least 30 somatic antigens and several surface components. The major problems are specificity and cross-reactivity of species within a family as well as with phylogenetically distant microorganisms [4]. Cross-reactivity also occurs in regions endemic for both leishmaniasis and trypanosomiasis. The use of recombinant antigens has improved sensitivity and specificity [7].

Most work concerning the use of immunoblotting for diagnosis of leishmaniasis has been carried out on VL. Although anti-leishmania antibodies are detectable in CL, they are present only at low titres; hence immunoblotting has not been widely adopted for diagnosis of CL [3].

Antigen detection is, in theory, an ideal method for diagnosing an infection, particularly since antigen levels are expected to correlate with the parasite load. However, although a few reports have been published, no satisfactory antigen detection system is currently available [8]. Despite the large number of serological tests available, there is no gold standard diagnostic test for VL, mainly because none of the tests are 100% sensitive and specific [3].

Molecular diagnostic methods include monoclonal antibodies (MAbs), DNA probes, and the polymerase chain reaction (PCR). MAbs and isoenzyme electrophoresis are undoubtedly the most precise methods for Leishmania identification, but these techniques are not applicable to the routine diagnosis of leishmaniasis. MAbs have the advantage of allowing the characterization of the parasite species using ELISA, IFAT or immunohistochemistry.

The successful use of a DNA probe depends on the target sequence. Examples are kinetoplast minicircle DNA (due to its high copy number, kDNA provides multiple targets for DNA probes and contains a conserved region of at least 120 base pairs), ribosomal RNA genes, minixenon-derived RNA genes or genomic repeats [3, 9]. PCR provides the most sensitive diagnostic technique for species specific genes. The PCR assay can detect parasite DNA or RNA a few weeks ahead of the appearance of any clinical signs or symptoms. Different DNA sequences in the genome of Leishmania, e.g. the ITS region, gp63 locus, telomeric sequences, and targets in rRNA genes such as 18s rRNA and SSU-rRNA, and both conserved and variable regions in kinetoplast DNA (kDNA) minicircles, are being used by numerous groups [10–13].

The best targets for PCR, as for DNA probes, are still the repetitive sequences. They are either based on kDNA minicircle conserved regions or complete minicircle amplification [14, 15], minixenon-derived RNA genes [16], small subunit ribosomal genes [17] or gp63 [18].

A recently developed combined PCR-ELISA technique has been reported to be sufficiently sensitive and specific for use as a diagnostic test in mucocutaneous leishmaniasis. PCR is perhaps the most reliable diagnostic method. However, it has not yet been developed for practical use in the field and awaits further improvement.

Real time PCR is also being applied as a molecular tool for diagnosis. However, this method is complex, requires many internal controls, and may also be too complicated and expensive for use in the field.

Both molecular and serological diagnostic methods rely on using single antigens or sequences that are specific enough to enable identification of the infectious agent. To date, methods for improving specificity and sensitivity are still being developed.

New approaches in molecular diagnosis: application of genome-wide technologies

High-throughput technologies introduce a new approach for diagnosis, namely the dimension of simultaneous analysis of many genes and/or proteins at the same time. The number of diagnostically relevant targets identified is growing. Paraphrasing Clare Fraser of The Institute for Genomic Research (Rockville, MD, USA) in her presentation at the Association for Molecular Pathology Meeting (Philadelphia, PA, 17 November 2001), “genomics has provided us with a parts list without the instruction manual”. In this context, genomics paves the way for the upstream search for diagnostics, vaccines and therapeutics, so that the results can be realized sooner [19].

The world of diagnostics and therapeutics is changing towards greater complexity, and the era of the ‘blockbuster drug’ may soon be over, making way for the ‘tailor made’ treatment regime. This ‘pharmacogenomic strategy’ requires screening and cataloging of patients’ gene and protein profiles prior to, during and following treatment, using efficient high-throughput technology [20]. Pharmacogenomics is unlikely to be practically useful or affordable for orphan diseases for the foreseeable future.

DNA microarray

DNA microarrays make it possible to analyse the
mRNA expression of thousands of genes simultaneously. The resulting comprehensive gene expression surveys lead to the identification of new genes and pathways with importance in cancer development and progression, or as targets for new therapies. Furthermore, some reference laboratories are carrying out 1800 molecularly-based cystic fibrosis tests per week [19]. A high-density microarray has been described that identifies 54 different Mycobacterium species, using 82 unique 16S rRNA sequences and all known mutations associated with rifampicin and isoniazid resistance in M. tuberculosis [21]. The reasons for this rapid increase are two-fold. First, the era of genomics has provided us with the sequences of important organisms including our own (Homo sapiens) as well as many bacterial and viral pathogens. The validation and prioritization of genes emerging from genome screening analyses in large series of clinical tumours has become a new bottleneck in research [22].

The current fashion for functional genomics has put the spotlight on microarray technologies that are capable of comprehensive, quantitative analysis of RNA expression, with the promise of a global approach to the quantification of gene expression [23]. However, there are two conflicting opinions on the value of microarrays: they are either promoted as an exploratory-driven replacement for hypothesis-driven biology [24]; or they are criticized as expensive fads that cannot substitute for the old-fashioned, low-throughput approach to experimental biology. Unquestionably, microarrays can reveal associations between gene-expression signatures and the biology and outcome of disease – for example, by identifying clinically significant subtypes of cancers. This has raised expectations that the expression profile of a particular cancer will help in clinical management of this disease [25].

Fundamental issues of protocol and platform comparability remain to be addressed. There is no standardization of protocols or reagents for sample acquisition, or RNA or cDNA target preparation. All procedural errors are compounded by continuing uncertainties in the normalization step. The expression of housekeeping genes, which are frequently used as internal standards, is variable. Therefore, validation of changes in expression in an unrelated, secondary assay remains a critical requirement, and is best accomplished using the real-time reverse transcriptase polymerase chain reaction (RT-PCR) with its high dynamic range, bearing in mind the limitations of the technique.

Recently, a tissue microarray (TMA) technology has been developed that efficiently tests the clinical relevance of candidate genes. TMAs are microscope slides containing samples from hundreds of individual tumour specimens. They can be used for large-scale, massively parallel, in situ analysis of genetic alterations on a DNA, RNA and protein level using in situ hybridization or immunohistochemistry on hundreds of tumour specimens at a time [22]. When using tissues, the accuracy of quantitative gene profiling is critically dependent on microdissection, especially on the laser capture microdissection (LCM) of individual cells or cell populations [26].

**Protein microarray**

DNA array work is predicated on the assumption that quantification of cellular levels of mRNA transcripts can function as a surrogate for measuring the expression or activity of the protein which is the final functional product of gene expression. However, the correlation between the number of mRNA and protein molecules is generally not strong enough to predict one value from the measurement of the other [27], as there is at least a ten-fold range of protein abundance for mRNAs of a given abundance [28]. Certainly, gene expression levels per se tell us very little about the biological function of a given abundance.

Microarrays are already having a major impact on cancer biology, pharmacology and drug development and, with their rapid development, new technologies, methods of analysis and applications are emerging continuously. However, the major limiting factor in their further application is the current lack of data comparability, which is essential for appropriate comparisons between different array data.

Furthermore, a full understanding of pathogenesis of an infectious agent could ultimately require knowledge of levels of proteins and their variants. Therefore, there is an increasing need to use and develop proteomics to explain complex disease phenotypes. Identification of all protein variants, how they relate to each other and the functional consequences of changes in their levels must be considered in order to understand the clinical presentation of complex diseases. Hence, protein arrays appear to be promising tools for drug screening and diagnostics.

Proteins can be arrayed either on flat solid phases or in capillary systems (microfluidic arrays [29]). Preferred solid phases are modified glass or filter membranes, because of their low-fluorescence background. Nitrocellulose and polyvinylidene difluoride membranes as well as polyamine and
polyaldehyde derivatized slides have also been used [20].

The concept of antibody arrays is derived from two sources: classical immuno-capture assays and cDNA arrays. First, high-throughput probing of hundreds of compounds in complex mixtures (e.g. body fluids or tissues) can be miniaturized. Second, robust, hybridization-like antibody-antigen binding can be used in a standard way to identify all or certain marker proteins expressed from a genome. Their common molecular features make antibodies ideal candidates for standard assay formats and the creation of protein-expression profiles, complementing and extending diagnostic options offered by DNA microarrays [30].

**Analyses**

As indicated above, the use of high-throughput technologies has lead to a new era in diagnosis. It enables screening and cataloguing of patients’ gene and protein profiles prior to, during and following treatment, using efficient high-throughput technology. Based on the concept of the arrayed library, cDNA clone arrays can be used for high-throughput expression and analysis of unknown proteins [31]. As a variant of this principle, antibodies can be screened and selected from phage display libraries using bacterial clone arrays [32]. Building upon established DNA microarray technology, arrays of purified proteins represent the next level diagnostic tools, allowing direct analysis of gene functions. Protein arrays enable miniaturized and highly parallel ligand binding assays for protein identification, quantification and affinity studies [33].

Protein affinity assays are used to analyse interactions between proteins such as antibodies, receptors or enzymes with other proteins, peptides, low-molecular-weight compounds, oligosaccharides or DNA. Protein profiles and interactions can already be correlated to states of cellular activity and disease [20].

Using new bioinformatics software (genetic algorithms and cluster analysis), proteomic patterns in serum were shown to completely discriminate cancer from non-cancer within the ovary [34]. These findings indicate that proteomic pattern analysis might ultimately be applied as a screening tool for cancer in high-risk and general populations. This also applies to autoimmune diseases, by screening sera of patients or high-risk individuals for the presence of specific autoantibodies, using arrays of large numbers of recombinant proteins of known identity [20].

So far, a microarray-based immunoassay involving 18 different autoantigens and high-throughput arrays featuring 196 distinct biomolecules and containing the major autoantigens of eight distinct human autoimmune diseases, including systemic lupus erythematosus and rheumatoid arthritis, have been reported. These are the first, but extremely promising, steps towards high-throughput protein arrays as diagnostic tools, eventually arriving at the doctor’s office and as over-the-counter applications [20].

Functional proteomics provides a powerful approach to screen for alterations in protein expression and posttranslational modifications under conditions of human disease [35].

Multidimensional chromatography of cell and tissue lysates or cellular subfractions could become an important alternative to recombinant protein expression. By consecutive chromatography and isoelectric focusing, cellular protein extracts have been directly fractionated, arrayed and detected with antibodies [36]. For example, protein screening to examine markers of melanoma progression, by profiling melanocyte versus melanoma cell lines using two-dimensional electrophoresis and mass spectrometry [35].

**Recommendations**

As indicated above, current methods of diagnosis of leishmaniasis include the parasitological and serological, using patient tissue smears from biopsy or sera against specific parasite antigens. The emergence of genetic engineering allows molecular diagnosis, which includes amplification of species-specific parasite sequences using the PCR. We recommend TDR support the application of DNA and protein microarray technologies for the diagnosis of leishmaniasis as well as for the study of parasite virulence, development and host-pathogen interactions as follows:

- The use of DNA microarrays for the identification of better and more species-specific markers. The following directions should be encouraged:
  - Comparative genomics of the various pathogenic *Leishmania* strains/species. These studies should elucidate all possible species-specific genes. In addition, their expression profiles during infection should be assessed.
  - Gene expression of host genes during infection. This can be done using either DNA microarrays of infected vs. non infected macrophage cell lines or with patient tissues. The results of these studies should provide important information on host-parasite interactions as well as the host reaction to parasite vir-
ulence. These results will also be useful for improved diagnosis.

- Proteomics is an additional tool to study gene expression. Since, regulation of gene expression in *Leishmania* is post-transcriptional, the appearance of specific proteins is critical for the study of pathogenesis, drug development and diagnosis. The following directions should be encouraged:
  - Identification and characterization of *Leishmania* proteins in all developmental stages, using state of the art methodology. This should also include post-translational modifications such as phosphorylation and glycosylation.
  - Comparative proteomics of pathogenic species.
  - Comparative analyses of gene expression, e.g. mRNA and protein abundance throughout the life stages of *Leishmania*. This includes the development of bioinformatic tools to compare mRNA with protein abundance in order to assess whether it is necessary to use both for diagnosis as well as studying development.
  - Development of protein microarrays of *Leishmania* proteins. This can be done by preparing a *Leishmania* expression library of the whole genome and expressing all in bacteria followed by printing the proteins/peptides on a solid phase, as has been recently done with yeast. This will be useful for the study of phenotypic expression in *Leishmania* and more importantly for diagnosis.

- Development of tools for genome wide-based diagnosis in the field for genomic and proteomic microarray analyses.

Ideally, the diagnostics that are developed should be sensitive and detect infection and distinguish between prior exposure and current infection. They should be specific and distinguish *Leishmania* from other pathogens and non-pathogens and distin-

### Table 1. Drugs used to treat visceral leishmaniasis and their principal limitations (references 42–45)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Structure</th>
<th>Main limitations or issues</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pentavalent antimonials</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium stibogluconate (Pentostam)</td>
<td>[Image of sodium stibogluconate structure]</td>
<td>Drug resistance (up to 65% in Bihar, India) Parenteral administration (i.m. or i.v.) Prolonged treatment (up to 4 weeks) Toxicity (liver and pancreas, especially in HIV patients) Poor compliance Cost (US$ 120–150)</td>
</tr>
<tr>
<td>Meglumine antimonate (Glucantine)</td>
<td>[Image of meglumine antimonate structure]</td>
<td></td>
</tr>
<tr>
<td><strong>Diamidines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4,4'-Diamidino-α,ω-diphenoxypentane: Di-isethionate salt (Pentamidine)</td>
<td>[Image of diamidine structure]</td>
<td>Parenteral administration (i.m.) Prolonged treatment (up to 4 weeks) Toxicity (myalgia, nausea, hypoglycaemia, diabetes) Poor response rates (around 75%) Cost (US$ 60–150)</td>
</tr>
<tr>
<td>Dimesylate salt (Lomidine)</td>
<td>[Image of Lomidine structure]</td>
<td></td>
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<tr>
<td><strong>Polyene antibiotics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>[Image of Amphotericin B structure]</td>
<td>Parenteral administration (slow i.v. infusion) Prolonged hospitalization Severe toxicity (hypokalaemia, renal failure, metabolic acidosis, cardiotoxicity) Cost (Fungizone US$ 60–150; AmBisome &gt; US$ 1000)</td>
</tr>
<tr>
<td>Desoxycholate formulation (Fungizone)</td>
<td>[Image of Amphotericin B structure]</td>
<td></td>
</tr>
<tr>
<td>Lipid liposomal formulation (AmBisome)</td>
<td>[Image of Amphotericin B structure]</td>
<td></td>
</tr>
<tr>
<td><strong>Aminoglycoside antibiotics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paromomycin sulphate (Aminosidine)</td>
<td>[Image of Paromomycin sulphate structure]</td>
<td>Parenteral administration (i.m.) Prolonged treatment (up to 3 weeks) Toxicity (nephrotoxicity, nerve deafness) Cost (US$ 50) Availability</td>
</tr>
<tr>
<td><strong>Ether lipid analogues</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexadecylphosphorylcholine (Miltefosine)</td>
<td>[Image of Hexadecylphosphorylcholine structure]</td>
<td>Toxicity (teratogenicity; contraindicated in pregnant women) Ease of resistance (long half life of drug) Patient compliance Cost (US$ 50)</td>
</tr>
</tbody>
</table>
guish parasites that are likely to cause serious or lethal disease from those that are likely to result in mild disease or be asymptomatic. They should be able to recognize drug resistant strains and assess the consequences of response to therapy, especially adverse responses such as post kala azar dermal leishmaniasis.

**DRUG DISCOVERY**

**Current anti-leishmanial drugs**

Current drug treatment of leishmaniasis is unsatisfactory (see Table 1 for a list of principal drugs and their main limitations). Of particular concern is the fact that the clinical value of the front-line pentavalent antimonial drugs (Pentostam and Glucantime) is seriously threatened by the emergence of widespread resistance in parts of India and Bangladesh [37]. In addition, treatment efficacy with many of the available drugs is reduced by immunosuppression, particularly in patients co-infected with HIV [38, 39]. Thus, new drugs are required primarily for the treatment of the life-threatening visceral form of leishmaniasis and secondarily for the disfiguring mucocutaneous and cutaneous forms of the disease.

The genome sequence provides a substantial opportunity for identifying novel potential drug targets through bioinformatics and database mining. Comparative genomic studies could identify biochemical targets that are common to *Leishmania* and trypanosomes, thereby reducing drug discovery and drug development costs of a broad spectrum anti-trypanosomatid drug. Many genes that are unique to *Leishmania* (or better still, are common with trypanosomes but absent from humans) have unknown functions that may be essential for parasite growth or survival. The essentiality/redundancy of such genes needs to be investigated by gene disruption or gene knockdown, and the biological or biochemical function of their products established. Target validation is a major bottleneck in drug discovery, and WHO/TDR could play a coordinating role in this post genomic activity. As summarized in Table 2, a powerful range of genetic tools are now available to address this question [46]. However, unlike African trypanosomes [40], *Leishmania* do not appear to be susceptible to RNA interference (RNAi), the current method of choice, possibly due to lack of some or all components of the RNAi machinery [41]. Thus, improved genetic methods for target validation need to be developed. WHO/TDR supports a num-

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**Table 2. Genetic tools in trypanosomatids (adapted from reference 46)**

<table>
<thead>
<tr>
<th>Genetic tools</th>
<th><em>T. brucei</em></th>
<th><em>T. cruzi</em></th>
<th><em>L. major</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro culture models</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>In vivo disease models</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Transient transfection</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Stable transfection</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Expression vectors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Episomal</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>- Integrating</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>- Regulatable</td>
<td>+++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Selectable markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Positive</td>
<td>6</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>- Negative</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Gene knockouts</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Sexual crossing</td>
<td>+</td>
<td>?</td>
<td>-</td>
</tr>
<tr>
<td>Positional cloning</td>
<td>Possible</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>RNA interference (RNAi)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Functional rescue</td>
<td>+</td>
<td>ND</td>
<td>+++</td>
</tr>
<tr>
<td>Transposon mutagenesis</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Genome size (Mb)</td>
<td>35</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Number of chromosomes</td>
<td>11 plus mini-chromosomes</td>
<td>&gt;30</td>
<td>~36</td>
</tr>
</tbody>
</table>

ND = not determined
*+, ++, +++ = the given method has been developed; the number of + symbols reflects an approximate assessment of how well the method works and/or the extent of its use.
number of centres carrying out small molecule screening against Leishmania and other parasites. Improved reporter methods to increase throughput in cellular assays are required to identify new classes of lead compounds with novel modes of action (chemical genomics).

The Leishmania genome also provides new opportunities to further define the mode of action of existing drugs, with a view to developing novel classes of drugs against existing targets or providing a scientific rationale for combination chemotherapy. Drug resistance is a serious threat to current therapy and a more detailed understanding of resistance mechanisms in clinical isolates may reveal novel strategies to circumvent drug resistance. The development of improved diagnostic methods could be useful in monitoring clinical responses to drug therapy and as a predictor of drug sensitivity in clinical isolates. At the present time, few compounds are in the late discovery or preclinical phases of development. WHO/TDR should be proactive in identifying suitable validated “druggable” targets and funding the development of robust, cheap and reproducible assay methods for high-throughput screening.

Recommendations

The following activities are recommended in order to translate genome information into drug discovery and development:

1. Target identification by database mining and metabolic pathway reconstruction (identify novel or unique/different pathways or enzymes common to trypanosomatids).
2. Target validation using reverse genetics (identify genes essential for amastigote growth/survival).
3. Development of inducible systems for conditional gene knockout and RNAi, possibly by knock-in of the dicer system.
4. Target characterization (establish biochemical function, molecular mechanism and structure).
6. Assay development and high-throughput screening (identify lead compounds).
7. Screening of lead compounds against whole cells (chemical validation of target; demonstration of selectivity for parasite versus host).
8. Comparative studies at the DNA, RNA and protein levels in drug sensitive and drug resistant lines (modes of drug action and drug resistance).

VACCINE DEVELOPMENT

Current anti-leishmanial vaccines

The immunity that follows recovery from Leishmania infection illustrates the feasibility of developing an effective vaccine; however, no such anti-leishmanial vaccine is available for practical use at this time. The protective immunity against Leishmania entails strong Th1 T-cell responses with associated IFN-γ and IL-2 production, while disease is associated with a Th2 response and production of associated cytokines. The outcome of infection depends on the balance between Th1 and Th2 responses, with the former being associated with disease resolution as well as protective immunity. Experimental mouse model systems have been useful in generating these insights, and generally parallel the immune responses and disease outcome in human infections. Mouse model systems have also been used to explore the development of anti-leishmanial vaccines. Characteristically these models employ strains of mice and parasites that differ in susceptibility and virulence, respectively. The host and pathogen strains are selected to mimic natural disease, which illustrates the importance of both host and pathogen factors in disease. As with any model system, care must be taken when extrapolating to the human host as well as to parasite strains that occur in the field. Studies to develop leishmanial vaccine have utilized antigens ranging from live parasites to subunit vaccines [47, 48]. These studies have illustrated that development of an effective vaccine also requires an appropriate immunization protocol and adjuvant to elicit the needed Th1 response.

Live vaccines utilizing species of Leishmania that cause cutaneous disease result in strong immune responses after healing of the lesions in humans. Attempts have been made to standardize the inoculum for reasons of reliability, safety, and efficacy [49]. Similarly, attenuated Leishmania that have been generated by genetic modification through elimination of gp63 protease [50] or DHFR-TS [51] genes have been examined in mouse model systems. Protection is achieved with these vaccines, although it is not fully analysed with respect to cross-species protection. However, the use of live or live attenuated parasites as vaccines is unlikely to be practical for reasons of safety and economics. Non-live complex vaccines consisting of killed cells or cellular fractions have also been explored as potential vaccines. These include immunization with killed L. amazonenesis, L. major, L. mexicana, and L. braziliensis alone or in combination; these were administered variously with BCG or cytokines as adjuvants for both prophylactic and therapeutic vaccines, and were tested in mice and dogs and in human trials. They appear safe and
elicit significant protection from natural infection in humans and animal model systems, although the proportion of protected humans and the utility of delayed-type hypersensitivity as a surrogate marker of protection is uncertain [47, 52, 53].

Several recombinant proteins have been assessed as vaccine antigens. Such antigens offer the obvious advantages of control of composition and reproducibility; however, they elicit a narrow immune response and hence their efficacy is less certain. The antigens tested include proteins encoded in multi-gene families such as the gp63 protease and the membrane antigen known as gp46/M2 or PSA2. Analyses in animal model systems have indicated species-specific differences in protection and potential importance of conformational epitopes. Other antigens tested as vaccine candidates include LACK, the homologue of receptor activated C kinase; Leishmania eukaryotic ribosome protein (LeIF), the homologue of an initiation factor 4A; TSA (trypanothione peroxidase), a homologue of a eukaryotic thiol-specific antioxidant protein; M15, a homologue of a yeast stress inducible protein; and LCR1, which has homology with a T. cruzi flagellar antigen. LeIF, TSA, and M15 have been tested alone and in combination. More recently identified vaccine candidates include amastigote specific antigens A2, P4 and P8. Synthetic peptides derived from the above antigens, as well as the non-protein antigen Leishmania lipophosphoglycan (LPG), induced protective immunity, but further study is needed. In general, these antigens vary in ability to induce protection, and this ability is often influenced by the adjuvant and immunization scheme. The protein antigens induce variable degrees of partial immunity with varying cross-species protection. The peptide antigens result in limited protection, while protection with LPG is more robust. Detailed assessment of these vaccine candidates must be considered as work in progress [54].

Identification of vaccine candidates entails various approaches, ranging from studies unrelated to vaccine development to various forms of immune interrogation of cellular or recombinant leishmanial proteins. DNA encoding these antigens has been used not only as an immunogen to stimulate T-cell responses but also as a method to screen numerous potential protein antigens [55]. Complexities arising from the importance of post-translational modification in inducing immunity and the possibility of inhibitory interactions among gene products potentially limit this approach. However, with the completion of the Leishmania genome sequence, the potential for a comprehensive survey of all possible Leishmania protein antigens is now feasible. The availability of appropriate assays, as well as the stability and economy of such vaccines, make DNA vaccines attractive. In addition, gene (and hence antigen) variation among strains and species is likely to be an important consideration for the development of vaccines that broadly cross-protect among strains and species. Analysis of such variation at the DNA level, as well as accommodating it in a DNA vaccine, is reasonably straightforward.

**Recommendations**

Substantial evidence exists for the ability to develop a vaccine, although the specific correlates of acquired and induced immunity are yet to be defined. Therefore, we recommend support for vaccine development with an emphasis on genome-wide approach. We suggest:

- Proteome-wide analyses (protein microarray studies) to identify protein antigens that are predicted to protect against infection or disease.
- Identification of amastigote-specific antigens in the entire proteome.
- Characterization of immune responses specific for natural or induced protection.
- Development of live attenuated (recombinant) parasite vaccines.
- Combined vaccine development and immunopathogenesis studies.
- Studies of epitopes that may cross-react between host and pathogen.
- Assessment of protection in mouse model systems with parallel human analyses.
- Identification of T-cell receptor associated peptide antigens (mass spectrometry), and reactivity to specific antigens by ELISPOT and protein arrays.
- Use of DNA vaccines as a tool for identifying vaccine candidates.
- Exploration of immunization strategies – prime/boost and adjuvant combinations.

Mouse model systems have been very fruitful and should continue to be used in combination with genetic manipulation of the pathogens and in concert with examination of human parallels to the experimental mouse model systems. Such studies will advance our understanding of factors that are critical for the development of disease and can lead to insights that could be used to develop preventative or therapeutic measures or for patient management.

**CONCLUDING REMARKS**

The near completion of genome sequencing for *L. major* and other kinetoplastids has provided us with an unparalleled wealth of information relating to
the biology of these clinically important parasites. The challenge we now face is how to rapidly and efficiently exploit this abundance of information in order to develop affordable new drugs, vaccines and diagnostic tools for the control of these greatly neglected diseases.

References


7.3 CAN LEISHMANIASIS STILL BE CONSIDERED A TDR CATEGORY 1 DISEASE? IF MOVED, WHAT IS REQUIRED?

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I would like to begin the assessment of research and capacity needs for the control of leishmaniasis with an authoritative view on science and problem solving from a giant of innovation:

“If we all did the things we were capable of doing, we would literally astound ourselves.” —Thomas Edison

THE CHALLENGES OF PREVENTION AND CONTROL OF LEISHMANIASIS

Endemic leishmaniasis is perceived as a problem of low priority by governments, society and patients themselves. Even though case detection and treatment are the primary control measure in most endemic settings, and leishmaniasis is a disease of obligatory notification in 33 of 88 endemic countries, passive detection and under-reporting prevail (Desjeux, 2004). Factors that contribute to the perception of low priority include:

- other or urgent health problems of greater magnitude or impact (medical, public health, political)
- limited resources (human, organizational, financial)
- lack of current information about the magnitude or nature of the problem
- absence of knowledge about the most effective points for intervention in the problem (as opposed to reacting to the ultimate, often epidemic, manifestation of the problem)
- ignorance in affected communities about the importance and availability of diagnosis and treatment.

In fact, leishmaniasis is not a health priority in most endemic countries, but it is important in all of them. What is important about leishmaniasis in individual countries can be very different, and if what is important can be identified, then this may become a priority even if the disease is not. The capacity to determine what is important about leishmaniasis in different settings is crucial to prevention and control and to mobilizing resources. For example, if case detection and treatment are important, then educational strategies for affected populations that promote understanding of the disease, the urgency of early diagnosis, and adherence to treatment are required, as are development and evaluation of point-of-care diagnostic tools, and the capacity to evaluate alternative less toxic oral or topical therapies. If vector control is a key issue, then the capacity to conduct entomological assessment of areas of transmission in order to target control measures and evaluate the cost-effectiveness and sustainability of the intervention are needed.

Worldwide, the epidemiological circumstances of Leishmania transmission vary widely (Desjeux, 2001). Moreover, the circumstances of transmission are continually evolving in relation to environmental, demographic and human behavioural factors (Lainson, 1989). Urbanization of transmission, spread of HIV co-infection, impact of land use on transmission, and internal and international conflict have changed the landscape of leishmaniasis. Data on, and knowledge about, these changes and their significance for control are scarce, almost anecdotal. Communication of the knowledge gained in investigating specific foci of transmission and interventions is often delayed for years, or not undertaken due to the arduous and unfamiliar demands of publication in national and international journals, language constraints and limited experience of endemic country scientists and control personnel in communicating through this medium. Alternative mechanisms and media for systematically communicating epidemiological trends, associated factors and results of interventions at the national and regional level are needed in order to make information opportunely available. National epidemiological bulletins along the lines of the United States Center for Disease Prevention and Control (CDC) Morbidity and Mortality Weekly Report (MMWR) might begin to address this information gap. Capacity building to implement such a medium of communication in developing countries could have a high payoff simply by making information available to public health authorities, investigators, and policy-makers. Likewise, development of capacity in epidemiological assessment, surveillance and reporting for disease control authorities would empower those on the front line to convert the results of their work into accessible information, which is fundamental to resolving the problem.

Accessible epidemiological information is necessary for integration of control measures. Information technologies such as geographic information sys-
tems have begun to be exploited in the surveillance of risk for outbreaks of leishmaniasis (King et al., 2004). Integration of leishmaniasis control with the control of other vector borne diseases could allow limited resources to be used more effectively, as has been proposed and widely recommended. However, this is not automatic, nor are data substantiating effectiveness and cost savings readily available. Investigating and planning how interventions for different vector borne diseases can be integrated, evaluating the effectiveness of integrated measures, and determining the savings generated, constitute requirements for successful and sustainable integration of leishmaniasis control with control of other vector borne diseases. Emerging problems such as yellow fever, dengue and West Nile virus can divert resources from other vector borne diseases such as leishmaniasis, but this is less likely to impair control if integrated programmes anticipate such circumstances.

**VACCINATION**

For quite a long time, it has been known that, in most cases, symptomatic natural infection of immunocompetent individuals with *Leishmania* of any species and in any epidemiological context leads to resistance (perhaps lifelong) against new disease. The practice of leishmanization derived from this empirical knowledge, and accessible data from some of the vast experience with leishmanization, substantiate the feasibility of inducing protective immunity by artificial infection (Gurges, 1971; Greenblatt,1980; Kellina, 1981; Nadim, Javadian & Mohebali, 1997). So the feasibility of vaccination against leishmaniasis seems self-evident. How to accomplish this is another issue. During the past two decades, thousands of research reports on leishmaniasis have been published, much has been learned, and the feasibility of immunoprevention has been experimentally demonstrated with a range of antigens, adjuvants and strategies of administration. Nevertheless, no vaccine candidate has approximated the protection afforded to humans by leishmanization.

Experience with leishmanization and with attenuated *Leishmania* in experimental models has suggested that pathogenicity (“virulence” as defined by the production of a lesion) is associated with protection. Avirulent strains either fail to induce protection or do so only partially. Immunotherapy with killed *Leishmania* has provided proof of principle that intervention of the immune response can reduce morbidity (Convit et al., 1987, 1989, 2004). Yet even successful immunotherapy using killed *Leishmania* has required BCG or multiple series of ten daily inoculations of antigen, apparently vindicating the adage “No pain no gain”. But is it pathogenicity, or capacity to complete the life cycle in the host, including persistence, that determines immunogenicity? Asymptomatic infections with *Leishmania* occur, and perhaps accumulate, in a variable proportion of the populations in endemic settings (Saravia, 1990; Weigle et al, 1993; Davies et al,1995); most of these remain asymptomatic indefinitely unless immune status changes. Hence pathogenicity does not appear to be essential to protection. Can targeted gene deletion extricate pathogenicity and leave protective immunogenicity intact? Deletion of genes encoding “virulence factors” while retaining infectivity and persistence in vivo, provides evidence that this may be achievable (Spath et al., 2003; Alexander et al.,1998). The feasibility of enhancing the immunogenicity of an attenuated live vaccine by recombinant genetics or exploitation of non-specific immune activation through CpG sequences (Rhee et al., 2002; Mendez et al., 2003) or other Toll receptor ligands to achieve healing is also supported by experimental and clinical evidence (Buates and Matlashewski, 1999; Arevalo et al., 2001).

What are the technical specifications of a live attenuated *Leishmania* vaccine? How will we know when we have a “viable” candidate? What is the appropriate “gauntlet” of evaluation for this or any vaccine? This information is lacking, yet would serve to focus research and development efforts at the preclinical and clinical stages of evaluation.

Safety, production including quality control, stability, and delivery are major concerns for all vaccines. Live vaccines, especially, provoke concerns of violating the Hippocratic oath (“first do no harm”) and of legal and financial liability, though other types of vaccine are not without risks. On the other hand, live vaccines contain the antigenic repertoire needed to induce protection in genetically heterogeneous populations, and engage the local, regional and systemic immune system. Furthermore, attenuated *Leishmania* may persist indefinitely (Alexander,1998; Streit, 2001), thereby maintaining a state of concomitant immunity. How do the potential benefits weigh in against the risks? While a molecularly defined subunit vaccine is being developed, are we missing the opportunity to prevent the serious consequences of leishmaniasis by failing to apply the lessons of leishmanization, the immunobiology of experimental leishmaniasis, the *Leishmania* genome and recombinant genetics, in designing a live, but eradicable, attenuated, non-revertible *Leishmania* vaccine? Alternatively, could DNA vaccines in a replicating vector approximate a live vaccine (Gonzalo et al., 2002)?
FIELD TRIALS AND CHALLENGE STUDIES

Protection of humans from disease is the ultimate criterion in evaluating a Leishmania vaccine. The availability of field sites with sufficiently high, predictable, incidence of new disease in the exposed population, and the accessibility of such high risk populations to follow-up, constitute significant constraints on the development of Leishmania vaccines. In addition, efficacy trials in endemic populations encounter the confounding potential of prior exposure to infection, even if skin test positive individuals are excluded, and are long. (requiring two or more years after safety and immunogenicity testing), involve large numbers of participants, and are very labour intensive. While challenge studies are highly demanding in terms of legal and ethical hurdles, knowledge of and strict adherence to good clinical practices (Hope and McMillan, 2004), and technical infrastructure, they can be conducted in small numbers of volunteers and provide definitive results in a short period of time (WHO/TDR Expert Review, 1997; Levine, 1998). Challenge studies could therefore, expedite the identification of candidate vaccines that induce protection in humans but would not replace field trials of candidates that prove effective in challenge studies. They would also allow correlates of protection to be identified by the evaluation of potential surrogate immune parameters prior to challenge and subsequent correlation with outcome (Levine, 1998).

The conduct of challenge studies in concert with leishmanization in endemic settings where leishmanization is still practiced requires political commitment, substantial investment in infrastructure, and technical and logistical assistance in the design, planning, execution, data analysis and evaluation inherent in this type of research. Ethical issues including informed consent in volunteer populations in developing countries, particularly the groups at high risk of leishmaniasis, are formidable. The experience gained in attempting to conduct Leishmania vaccine trials in conjunction with leishmanization in Uzbekistan and Iran offers invaluable lessons for building challenge study capability.

Capacity building to establish challenge study capability would require:

- apprenticeship training at postdoctoral level, of highly qualified scientists from disease endemic countries, in vaccine development centres where challenge studies are safely and rigorously conducted
- scientific and technological infrastructure commensurate with the task, i.e. established insti-

What about challenge studies via needle inoculation of volunteers in unexposed populations? Are these ethically and technically feasible in either developed or developing countries? Would the development of genetically attenuated Leishmania with drug susceptibility to a non-toxic, restricted-use antibiotic, or other genetic marker that permitted eradication, improve the ethical and technical feasibility of challenge studies? Does the molecular genetic technology required to create a minimally pathogenic, eradicable, protectively immunizing live vaccine exist? In-depth, authoritative analysis of these questions in the light of technological advances is needed to rally the scientific community for the daunting task of challenge studies.

LESSONS FROM OLD DRUGS, RESISTANCE, AND NEW DRUGS

The toxicity and high cost of the most effective drugs impose constraints on the treatment of leishmaniasis. In consequence, many patients in disease endemic countries do not receive adequate treatment. Avoidance of over-treatment is a major concern. This results in reluctance to treat based on clinical diagnosis and referral to centres equipped to conduct parasitological diagnosis in order to receive treatment. The availability of the oral drug miltefosine for visceral leishmaniasis (VL) could change the management of treatment so that serology, which is highly correlated with clinical VL, or the detection of specific antigen in urine (Attar et al., 2001), and standardized clinical criteria would be sufficient to administer treatment, thereby allowing treatment to be administered closer to home.

The identification and licensing of the oral drug miltefosine for the treatment of visceral leishmaniasis caused by L. donovani in India is a major achievement. Implementation of the use of miltefosine, and other drugs in the future, requires regulation and education to prevent misuse and loss of effectiveness. The efficacy of this drug for other species of Leishmania causing visceral disease, L. infantum/chagasi, L. amazonensis, and dermatotropic species in different geographic regions, is still uncertain. Nevertheless, if this drug proves to be widely effective against viscerotropic and dermatotropic leishmaniasis, disease control through active case detection and treatment would become possible.
The teratogenicity of miltefosine, however, presents a challenge in the design, execution and management of ambulatory treatment in order to avoid inappropriate use. Widespread use of any monotherapy in circumstances of anthropogenic transmission creates vulnerability to loss of susceptibility to the drug. Adherence to therapy is crucial. Therefore health education strategies to promote this and other personal and community protection methods constitute a priority need.

In addition to proper administration of available drugs, surveillance for the emergence of drug resistance and the use of combined regimens have proven protective of drug utility in other microbial infections. Combinatorial therapy is now recommended for treatment of *P. falciparum* malaria (Nosten and Brasseurs, 2002); similar strategies may be useful in conserving the life of miltefosine and other new chemotherapeutic options. Knowledge of the efficacy, pharmacokinetics, and desirable and undesirable interactions of drugs amenable to combination will be needed in order to rationally identify combined therapies. Since lifelong persistence of infection occurs, even after prolonged treatment with antimonial drugs, knowing whether miltefosine eradicates *Leishmania* or reduces the transmissibility of persistent parasites will be an asset in judiciously managing the use of this drug in the treatment, prevention and control of leishmaniasis. A growing range of reporter systems (green fluorescent protein, luciferase *luc* gene) and molecular amplification methods are now applicable to the experimental and clinical assessment of persistence of parasites post-treatment, and researchers in endemic countries are able to address these questions. Concerted protocol development and conduct of studies can promote the development of scientific community and bridge the gulf that frequently separates disease control authorities and research communities. Problem solving capacities, rather than promotion of competition for limited public resources, can be developed within the framework of such cooperative goal oriented endeavours.

Epidemic anthropogenic leishmaniasis and treatment failure linked to drug resistance have underscored the crucial importance of knowing whether parasites are eradicated or reduced to levels below the threshold of transmissibility by any individual or combined treatment including immunotherapy. If treatment with miltefosine, or any other safe and easily administrable drug, definitively and durably interrupts transmission, this knowledge could open the way for “mass” or community-based treatment. Then, individuals with scars from previously untreated leishmaniasis or, conceivably, even latently infected asymptomatic residents in emerging or expanding foci, could be considered for treatment. Furthermore, non-toxic oral or topically administered drugs theoretically reduce the need for etiologic diagnosis and medical supervision of treatment, since over-treatment would then be less of a concern than failure to treat or inability to make an etiologic diagnosis at the point of care. If “mass” treatment was found to be a technically feasible control strategy, as in the case of onchocerciasis and HIV, such an intervention would require consideration of cost vs. benefits and the possibility of increased risk of generating resistant organisms.

Treatment of HIV co-infection with *Leishmania* presents special challenges. Although in experimental murine models amphotericin does not require an intact immune response, as do pentavalent antimonial drugs, patients with HIV co-infection eventually relapse following treatment with amphotericin (Alvar et al., 1997; and personal communication). Resistance to multiple drugs can develop and could be transmissible among intravenous drug users or by sandflies. More effective drugs and therapeutic strategies are needed to address this expanding problem. Antiretroviral therapy has been shown to reduce the incidence of VL in HIV-positive populations (De La Rosa et al., 2002), and hence is a key consideration in the prevention and control of HIV co-infection.

All available anti-leishmanial drugs are costly in relation to the financial capability of affected countries and populations. Drug production in developing endemic countries has already lowered the cost of antimonial drugs; other drugs such as liposomal amphotericin formulations could be more accessible to the budgets of high burden countries if production could be undertaken in a developing country. Although few developing countries are in a position to undertake drug research and development or have production capacity for drugs, technological transfer and capital investment could empower some to generate long-term solutions (Trouiller et al., 2002). Moreover, production of biologicals and drugs could generate sustainable technological and research capacity since the lower cost of production and unsatisfied volume of demand would yield assets that could be reinvested in technological capacity and otherwise support economic development.

**IMMUNOTHERAPY**

Jacinto Convit in Venezuela pioneered immunotherapeutic approaches to dermal leishmaniasis, unequivocally demonstrating the plausibility of
inducing clinical cure by activating and presumably modifying the ongoing host immune response (Convit et al., 1987, 1989, 2004). Although repetitive injection of leishmanin and BCG is effective in promoting healing of lesions, the adverse effects of BCG in a variable but concerning proportion of patients, and the need for multiple parenteral inoculations over an extended period of time, have discouraged widespread general use of this approach to treatment. Multiple intralesional injections of killed *Leishmania* vaccine have also shown evidence of therapeutic benefit when administered with antimonial drug (Machado-Pinto, 2002), but this strategy is impractical for widespread use. Advances in understanding of the innate immune response have yielded innovations in topically applicable immunomodulatory drugs that can shift a Th2 to a Th1 type response. Imiquimod and related compounds act through Toll-like receptor 7. These compounds have shown promise as coadjuvants in the treatment of dermal leishmaniasis with antimonial drugs (Arevalo et al., 2002; Buates et al., 1999), whose anti-leishmanial efficacy is dependent on immunocompetence. Therapeutic gain is currently under investigation in controlled clinical trials. Nevertheless, topical agents offer practical advantages and would surely be a more acceptable and feasible therapeutic strategy than injectable formulations.

Immunotherapeutic application of vaccines developed for immunoprophylactic use provides a comparatively rapid indicator of disease-modifying immunogenicity while potentially offering coadjuvant benefit. Specifications for immunotherapeutic interventions and consensus guidelines for evaluation are needed, and would provide a basis upon which to compare different products and regimens.

**DIAGNOSTICS**

Despite noteworthy progress represented by the direct agglutination test (DAT) and K39 dipstick serology for visceral leishmaniasis, simple, inexpensive, parasitological, diagnostic tools usable by non-professional health workers in endemic areas are still needed. Together with less toxic therapeutics, such diagnostic tools would allow treatment and monitoring of treatment to occur close to the home and to be supervised by non-physician medical personnel. This in turn would increase access to appropriate medical care and lower its cost. Antigen detection in urine may address this need; however, field testing in different settings of zoonotic and anthroponotic VL are required to determine the sensitivity, specificity and robustness of this method.

The availability of an effective oral drug increases the incentive for early diagnosis. Since antibody titres and antigen excretion are generally lower in the early stages of VL, serological tests and antigen detection methods that are so useful at later stages may be less sensitive for early diagnosis. In consequence, each advance redefines the nature of the problem as well as its management.

**NUTRITIONAL INTERVENTION**

Malnutrition has been linked to the development of VL. Nevertheless, the role of nutrition, young age, and immunological maturity are not clear (Dye and Williams, 1993). Since VL usually occurs in highly vulnerable individuals in the context of multifactor distress, prevention and control strategies might include “vulnerability reduction” by amelioration of risk factors such as malnutrition. Rather than examining malnutrition as a risk factor, might we instead examine nutritional supplementation and health education as protective factors?

**SOME MACRO CONSIDERATIONS OF PREVENTION AND CONTROL**

Awareness of emerging and expanding foci of leishmaniasis at an early stage is crucial to prevention and control. Likewise, understanding of the situation and the intervenable circumstances precludes effective action based on available diagnostic, treatment and vector/reservoir control strategies. Timely, accurate information is generally not available for the control of leishmaniasis; we seem to have a substantial knowledge base about *Leishmania* and leishmaniasis but have not yet brought the collective knowledge to effectively bear on the diverse problems that each outbreak, or novel expression of the mutability of the host/parasite interaction, presents. Bednets, insecticides, personal protection, dog collars, and environmental management have all demonstrated some utility in controlling transmission in different circumstances, yet leishmaniasis is a growing problem. Hence we could probably say that, in fact, “existing tools have not been used effectively”. However, perfect tools for every situation do not exist, so we can also say that, in the case of leishmaniasis, while “existing tools are inadequate”, others, such as a vaccine, are non-existent. We must also recognize that, even if we have learned quite a lot, we do not have sufficient knowledge of the multiple diseases that constitute the leishmaniases to design an effective vaccine, develop a portfolio of drugs, or intervene effectively in every circumstance.

Knowledge brokering is a new theme and career opportunity in the global knowledge-based economy. Screening, organizing and reconfiguring infor-
mation is fundamental to mediating communication with groups having different objectives, i.e. public health authorities and investigators, communities and policy-makers. The ability to identify aspects of health policy and management that research can resolve is another essential feature of knowledge brokering. Knowing what people need to know, and being able to find that information through research, boils down to being responsive to demand or the “market” for research. The ability to find and recognize evidence produced by research builds the credibility needed to convene different groups to interact and thereby articulate demand for knowledge and the supply chain to fulfill that demand (Oldham and McLean, 1997). The skills of knowledge brokering are especially needed by health researchers in disease endemic countries in order to assure the effective use of available tools, to design better, more acceptable, cost-effective strategies, and, ultimately, to foster political will.

In conclusion, leishmaniasis is still a Category 1 TDR disease, but we have a huge inventory of assets. The challenge is to insightfully and strategically apply what we know, as well as continue to build the knowledge base to more efficiently and effectively detect, treat, and prevent this disease.

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