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Recent developments leading toward a paradigm switch in the diagnostic and therapeutic approach to human leishmaniasis

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Abstract

Purpose of review—To identify recent papers showing how human and parasite genetics influence leishmaniasis, and how understanding of the immunopathology may be utilized in immunotherapy for these diseases.

Recent findings—Progress has been made in recent years showing the complexity within populations of *Leishmania spp*. and indicating that different strains lead to diverse clinical pictures and responses to treatment. Thus detection of parasite genetic tags for the precise identification of infecting strains, and for predictive diagnosis of clinical and therapeutic fates seems now possible. Host genetic loci involved in disease outcome have been detected, which may also be explored for better case management. These developments in diagnosis will demand expanding the therapeutic arsenal to take their expected effect. This is starting to be fulfilled by immunotherapies successfully employed to treat cases refractory to standard first line drugs, as the result of a more profound comprehension of the immunopathology of the leishmaniases.

Summary—The knowledge mounting has already helped explain why different patients present different forms of leishmaniasis and respond differently to treatment, and may be on the verge of catalyzing a major change in the already over a century old paradigm of diagnosing and managing these patients.

Keywords

immune response; leishmania; leishmaniasis; polymorphism; treatment

Introduction

Leishmaniasis refers to a widely divergent constellation of disease syndromes caused by the *Leishmania* spp. protozoa. The extracellular promastigote form of leishmania is inoculated into humans by a sandfly, after which it undergoes phagocytosis by a mammalian macrophage and transforms to an intracellular amastigote. Major forms of leishmaniasis include cutaneous, mucosal and visceral leishmaniasis although many variations occur. Differences in clinical manifestations of leishmaniasis are explained only in part by the species of organism causing the infection [1]. Many publications delineate the murine and human immune responses during leishmaniasis. However, an understanding of host immunity alone is unlikely to unveil how a single genus is able to cause such a

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heterogeneous group of diseases. Understanding of the contributions of host and parasite genetic polymorphisms to the spectrum of the leishmaniases may provide insight into disease immunopathogenesis, providing the information needed to make immunotherapy a feasible choice within the therapeutic arsenal against these microorganisms. This review summarizes recent developments in immunity, host genetics and parasite variability that could translate into future approaches to disease management.

The type one response is key to immunity and pathology in leishmaniasis

A type 1 immune response is necessary to control leishmania multiplication and dissemination in humans. In contrast, interleukin (IL)-10 and transforming growth factor (TGF)-β facilitate leishmania proliferation. Promastigotes stimulate interferon (IFN)-γ production by natural killer (NK) cells [2], and IFN-γ in turn activates macrophages to kill leishmania. Even so, progression of leishmaniasis with extensive pathology occurs even in the presence of high levels of IFN-γ, especially in mucosal leishmaniasis [3,4]. Peripheral blood mononuclear cells (PBMCs) from individuals with visceral leishmaniasis and diffuse cutaneous leishmaniasis (DCL) produce low IFN-γ levels when stimulated with soluble leishmania antigen (SLA), allowing parasite multiplication and progressive disease [5]. Nevertheless, IFN-γ is detectable in sera of visceral leishmaniasis patients, suggesting the cytokine is produced by T cells in some body sites [6] but does not control leishmania growth. IL-10 and TGF-β play critical roles in downmodulating type 1 responses during visceral leishmaniasis and DCL, and IFN-γ production by PBMCs is restored *in vitro* by neutralization of IL-10 [5,7]. Interestingly, splenic T cells producing IL-10 are not CD4+ CD25+ (Foxp3) regulatory cells in Indian visceral leishmaniasis patients [6]. It was recently shown that IL-10 and TGF-β play important roles in the development of post-kala-azar dermal leishmaniasis (PKDL) [8]. Treatment of Indian visceral leishmaniasis patients with amphotericin B enhances IFN-γ and lowers IL-10 and TGF-β levels, and these treated patients rarely progress to PKDL. However, there are some patients in whom IL-10 and TGF-β levels remain high despite treatment with antimony; these individuals often experience reactivation in the form of PKDL [8].

During the earliest phase of localized cutaneous leishmaniasis due to *Leishmania braziliensis*, low levels of IFN-γ are observed whereas IL-10 is prominent. IL-10 plays a role in parasite persistence [9]. As the infection progresses, all clinical forms of *L. braziliensis* infection demonstrate antigen specific production of IFN-γ that predominates over IL-10 levels [10]. Leishmania-induced type 2 cytokines are usually low in concentration during both cutaneous leishmaniasis and mucosal leishmaniasis [9]. Even patients coinfected with *L. braziliensis* and helminths develop a predominant and exaggerated type 1 immune response to leishmania antigens, although these individuals take longer to heal than those without worms [11]. This delay to healing may be in part due to higher levels of IL-10 produced by these individuals.

Although a type 1 response is critical to control the leishmaniases, a loss of appropriate modulation of this response underlies the immunopathology of both visceral and tegumentary leishmaniases. During visceral leishmaniasis, the host loses or suppresses an appropriate type 1 immune response. In the case of tegumentary leishmaniasis, a strong type 1 immune response results in an intense inflammatory response and high expression of nitric oxide synthase (NOS) type 2 in active lesion sites. Late lesions progress toward cure and show only focal inflammation, with a decrease in $CD8^+$ and increase in $CD4^+$ T cells associated with the onset of fibrosis [12]. *Leishmania*-specific $CD8^+$ T cells are generated during infection and are important for IFN-γ production and cytotoxicity against leishmania-infected macrophages [13], but they may also contribute to the immunopathology of leishmaniasis. Antigen-responsive $CD8^+$ cells are in part responsible

for the exaggerated type 1 immune response detected during cutaneous leishmaniasis and mucosal leishmaniasis [14]. These cells do not adequately respond to immunomodulation [3,5], possibly due to a decrease in the expression of IL-10 receptor on their surfaces [14]. Moreover, upregulation of IFN-γ production may stimulate the FAS/FASL pathway and induce apoptosis of keratinocytes with resultant ulcer formation [15]. Overall, these findings underscore the need for different strategies to control the type 1 immune response as a goal of treatment of the different clinical forms of leishmaniasis. During visceral leishmaniasis the goal would be a boost, whereas during tegumentary leishmaniasis the goal would be a partial downmodulation of type 1 cellular immunity.

Host genetic factors contribute to outcome in leishmaniasis

An expanding literature is documenting associations between human genetic loci influencing immunity and the different clinical forms and outcomes of leishmaniasis. Recent studies of the different disease phenotypes and outcomes suggest that genetic factors indeed contribute significantly to the outcome of leishmaniasis. Familial aggregation of visceral leishmaniasis in Brazilian populations suggested that genetic factors contribute to both disease and the development of a delayed-type hypersensitivity (DTH) skin test, a marker of cured symptomatic or asymptomatic infection [16–19]. Candidate gene studies indicated associations between polymorphic alleles of *SLC11A1* (formerly called *NRAMP1*), *IL4*, *IFNGR1*, and *TNFA* (encoding TNF-α) and development of visceral leishmaniasis, asymptomatic infection, or PKDL [20–23]. In addition, several genome-wide scans have been performed using cohorts exposed to the *Leishmania* species causing visceral leishmaniasis. Bucheton *et al.* [21,24] reported linkage between a major locus on chromosome 22q12 and visceral leishmaniasis in one ethnic group in a village in the Sudan. However, a separate study documented linkage between visceral leishmaniasis and major loci on chromosomes 1p22 and 6q27 but not the chromosome 22 locus, in two Sudanese villages geographically near the Bucheton cohort, but representing different ethnic backgrounds [25]. Cohorts in these villages could be stratified by Y-chromosome markers indicating there were extended families originating from two male founders in this patriarchal society. Refined linkage analysis stratified according to the Y chromosome haplotype revealed the peaks of linkage originated from one male founder of one village population. These data suggest that analysis of extended pedigrees originating from a single founder, in this case a male founder marked by Y-chromosome haplotype, can reveal susceptibility loci influencing a subset of the entire population [25]. Although the Sudanese populations studied above were infected with *Leishmania donovani*, visceral leishmaniasis in Brazil is caused by the species *Leishmania chagasi infantum* [26]. A genome-wide scan of a population in northeast Brazil suggested regions on chromosomes 15 and 19 were possibly linked to the asymptomatic infection (i.e. protection against visceral leishmaniasis), whereas a distinct region on chromosome 9 was possibly linked to visceral leishmaniasis [27].

Genetic studies of tegumentary leishmaniasis have focused on association studies of candidate genes, in part because the more sporadic epidemiology of these infections is not conducive to large family studies, and because mucosal leishmaniasis is rare making family-based studies difficult. Alleles of the *TNFA* locus are associated with both mucosal leishmaniasis and cutaneous leishmaniasis [28]. Analysis of the IL-10 819 polymorphism showed that the CC allele is associated with higher levels of IL-10 than the CT or TT genotypes, and with an increased risk of cutaneous leishmaniasis [9]. In a Brazilian population at high risk for *L. braziliensis* infection, an IL-6 174 G/C promoter polymorphism was found to be strongly associated with susceptibility to mucosal leishmaniasis but not to cutaneous leishmaniasis [29]. A more thorough understanding of polymorphic host loci that contribute to the subtle determinants of the immune response to this genus of parasites could help explain the individual variability in disease outcome.

Parasite strains as determinants of clinical and therapeutic outcomes of leishmaniasis

Treatment failure is often caused by a lack of appropriate patient's adherence to first and second line drugs, but there is also a steadily growing body of literature indicating that *Leishmania* spp. is developing resistance to the most common drugs used to treat the disease, the antimonials [30,31]. Drug resistance is a well recognized problem in Indian visceral leishmaniasis, inspiring treatment trials of new medications [31–34]. Failure of antimony treatment of tegumentary leishmaniasis, the only current measure of resistant parasite strains, has come from highly affected countries such as Iran [35], Peru [36•] and Brazil [37,38]. Current population shifts due to military activities in the Middle East have greatly changed the prevalence of disease, and it is likely that other regions of resistance will emerge in the near future.

Drug resistant strains are being reported amongst evolutionarily distant species (e.g. *Leishmania tropica*, *L. donovani* and *L. braziliensis*) [35,36•,37,38,39•], suggesting that parasites are capable of adapting to drug pressure through parallel and diverse mechanisms. Recent data show that, at least among *L. tropica* strains, resistance to one antimony based drug confers crossresistance to antimonials but not other drugs [40•]. Furthermore, reports have emphasized that susceptibility to antimonials varies markedly among species and even between geographically distant strains of a same species of *Leishmania*, emphasizing the role of parasite polymorphisms on antimony resistance/susceptibility [35,36•,37,38,39•]. A recent report showed that the glycoproteins collectively called proteophosphoglycans (PPGs) are overexpressed on the surfaces of promastigotes and amastigotes of stibogluconate resistant clinical isolates of *L. donovani* from India [39•]. It was also observed that the redox active molecule trypanothione, which serves as the major low molecular weight thiol of the *Leishmania* spp., underlies antimony resistance of *Leishmania tarentolae* isolates [41]. As there are currently no markers of drug resistance amongst parasite isolates, it would be of great value to explore the use of PPGs and thiol levels for this purpose.

Several recent studies address molecules that confer parasite resistance to oxidant stress. Arginine metabolism is a determinant of parasite killing or survival in macrophages. Macrophages are stimulated to undergo classical activation and express iNOS by the type 1 cytokines IFN-γ and TNF-α, or stimulated toward alternative activation by the type 2 cytokine IL-4 promoting expression of macrophage arginase [42]. Arginine metabolized by iNOS produces the potent leishmanicidal molecule nitric oxide, whereas metabolism of arginine by arginase results in production of L-ornithine and other polyamines that are essential for intracellular leishmania growth [43]. Recently, it was recognized that leishmania parasite-encoded arginase also contributes to the local macrophage arginine concentration, influencing the macrophage response. Infections in mice inoculated with *L. mexicana* genetically lacking both alleles of the gene encoding arginase were significantly attenuated in comparison to infections with both wildtype and add-back control parasites. Arginase knock out parasites were defective in their ability to survive intracellularly in macrophages. Importantly, however, this intracellular growth defect was corrected when arginase knockout parasites were grown in macrophages from mice lacking the iNOS gene. This suggests that parasite arginase utilizes and depletes host cell arginine pools, diminishing the amount of substrate available to iNOS for generating NO•. Parasites lacking arginase, therefore, allow arginine concentrations to accumulate, enhancing the activity of iNOS and increasing the generation of the potent leishmanicidal molecule NO• exclusively in host macrophage that expresses iNOS [44•].

Consistent with the previous report, a recent report showed that clinical isolates of *Leishmania amazonensis* and *L. braziliensis* may exhibit resistance or susceptibility to NO•-mediated microbicidal activity. Isolates that were resistant to NO• *in vitro* were derived from human cases of disease with poorer outcomes [45]. These data are also consistent with previous and recent reports showing that the insulin-like growth factor I (IGF-I) promotes in-vivo and in-vitro growth among different species of *Leishmania*, acting directly on the parasites, at least in part, via activation of their arginase [46•].

Resistance to intracellular hydrogen peroxide is another phenotype implicated in progressive leishmaniasis [47]. It was recently reported that clones of *Leishmania guyanensis* capable of metastasis in golden hamsters contain cytoplasmic peroxiredoxin and peroxidase activities that differ from those of nonmetastatic parasites [48]. Also, laboratory strains of *L. guyanensis* that are capable of metastasis contain different isoforms of the molecules tryparedoxin peroxidase and elongation factor-1β compared with those of nonmetastatic *L. guyanensis* strains [49]. The tryparedoxin/tryparedoxin peroxidase system is a critical counterpart of the trypanothione–trypanothione reductase system, allowing parasites to both reduce and oxidize the low molecular weight thiol trypanothione and in this manner evade oxidative killing [41,50].

Several previous reports underscore the major role of parasite polymorphism on infection outcome. One goal of such research is to identify markers that would allow us to track different strains and improve epidemiologic detection of outbreaks as well as case management. Epidemiological studies have also indicated that parasite genotype influences disease even at the intraspecies level. Two reports from Colombia [51,52] described an increased frequency of mucosal involvement among humans infected with particular *L. braziliensis* zymodemes or strains. Another more recent report described a multiclonal population of *L. braziliensis* from a region endemic for tegumentary leishmaniasis in northeastern Brazil, and also reported a statistically significant association between distinct parasite genotypes (clades) and the clinical outcome (i.e. cutaneous leishmaniasis, mucosal leishmaniasis or disseminated leishmaniasis) [53].

Studies of Old World *L. donovani* infections have shown that development of PKDL is, at least in part, strain dependent. Such parasite strains may be differentiated by the use of genetic tags [54,55]. Recent developments have even indicated that some genes are significantly overexpressed among isolates from patients with PKDL compared with those obtained from cases of visceral leishmaniasis [56].

Immunotherapy as an emerging option for treating cutaneous leishmaniasis and mucosal leishmaniasis

There is still no effective vaccine against human leishmaniasis. Human trials using killed parasites and recombinant proteins for vaccination have resulted in only short-term protective immunity [5,10]. Only a few advances have been made in the treatment of these disorders over recent decades. The pentavalent antimonials are no longer practical as a standard treatment for Indian kala-azar due to high failure rates, and concerns have also arisen about resistance in cutaneous leishmaniasis and mucosal leishmaniasis [36•].

Miltefosine and aminosidine (paromomycin) are promising treatment options being pursued [57,58]. Furthermore, immunotherapy combined with standard antimony treatment has been successfully tested in cutaneous leishmaniasis and mucosal leishmaniasis. The immuno-modulatory options are based upon the rationale that a T helper type 1 (Th1)-mediated inflammatory response causes the pathology of tegumentary leishmaniasis. In a double blind controlled trial, addition of the TNF-α inhibitor pentoxifylline to standard antimony

treatment was more effective than antimony and placebo, reducing the healing time of mucosal lesions (Fig. 1) [59]. Similar findings were reported for patients infected with *Leishmania major* [5]. Several studies provide evidence that granulocyte macrophage colony-stimulating factor (GM-CSF) given in conjunction with antimony is more effective and leads to shorter healing time of the cutaneous ulcers than antimony and placebo [60,61]. Many studies are needed to test the efficacy of these already-tried immunotherapeutic approaches as well as other new and promising therapies to determine whether they are effective against infection with the different forms and species of *Leishmania*.

Conclusion

The diagnostic approach to leishmaniasis has remained unchanged over the last century. Studies of parasite and host genotypes are revealing several tiers to the complexity of leishmaniasis. Data summarized in this review suggest that the experimental basis is emerging that could lead to new paradigms in the approach to diagnosis and management of these diseases. Precise identification of parasite strains and correlation with clinical outcome could allow more meaningful approaches to treatment regimens. Identification of host loci that influence immune responses to infection might prove helpful in identifying new immunotherapeutic approaches and causes of treatment failure. New drugs and new approaches to immunotherapy could provide the greater array of therapeutic options that is critically needed to control this polymorphic group of diseases.

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• • of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 556–558).

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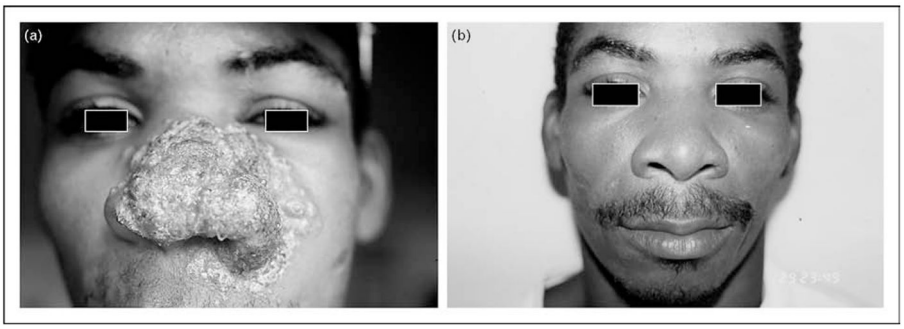

Figure 1. Immunotherapy is a promising new tool in the arsenal to fight leishmaniasis

(a) Facial lesion of a disseminated leishmaniasis patient after the fourth consecutive course of standard treatment with intravenous Glucantime at 20 mg/kg of body weight for 30 days. (b) The same patient after a single course of Glucantime (20 mg/kg of body weight) as in (a), combined with oral pentoxifylline at 400 mg, three times a day for 30 days.