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Review article

Leishmania model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity

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Abstract

Leishmanial mechanisms of virulence have been proposed previously to involve two different groups of parasite molecules. One group consists of largely surface and secretory products, and the second group includes intracellular molecules, referred to as 'pathoantigens'. In the first group are invasive/evasive determinants, which protect not only parasites themselves, but also infected host cells from premature cytolysis. These determinants help intracellular amastigotes maintain continuous infection by growing at a slow rate in the parasitophorous vacuoles of host macrophages. This is illustrated in closed in vitro systems, e.g. *Leishmania amazonensis* in macrophage cell lines. Although individual macrophages may become heavily parasitized at times, massive destruction of macrophages has not been observed to result from uncontrolled parasite replication. This is thus unlikely to be the direct cause of virulence manifested as the clinical symptoms seen in human leishmaniasis. Of relevance is likely the second group of immunopathology-causing parasite 'pathoantigens'. These are highly conserved cytoplasmic proteins, which have been found to contain *Leishmania*-unique epitopes immunologically active in leishmaniasis. How these intracellular parasite antigens become exposed to the host immune system is accounted for by periodic cytolysis of the parasites during natural infection. This event is notable with a small number of parasites, even as they grow in an infected culture. The cytolysis of these parasites to release 'pathoantigens' may be inadvertent or medicated by specific mechanisms. Information on the pathoantigenic epitopes is limited. T-cell epitopes have long been recognized, albeit ill-defined, as important in eliciting CD4+ cell development along either the Th1 or Th2 pathway. Their operational mechanisms in suppressing or exacerbating cutaneous disease are still under intensive investigation. However, immune response to B-cell epitopes of such 'pathoantigens' is clearly futile and counterproductive. Their intracellular location within the

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parasites renders them inaccessible to the specific antibodies generated. One example is the *Leishmania* K39 epitope, against which antibodies are produced in exceedingly high titers, especially in Indian kala-azar. Here, we consider the hypothetical emergence of this pathoantigenicity and its potential contributions to the virulent phenotype in the form of immunopathology. Microbial virulence may be similarly explained in other emerging and re-emerging infectious diseases. Attenuation of microbial virulence may be achieved by genetic elimination of pathoantigenicity, thereby providing mutants potentially useful as avirulent live vaccines for immunoprophylasis of infectious diseases.

Keywords: *Leishmania*; Macrophage; Infection; Microbial virulence; Pathoantigenicity; Live vaccine; Leishmaniasis

1. Introduction

Virulence is defined here as the degree of pathogenicity of a microorganism genetically endowed with that capacity, as manifested against a host with an intact immune system under normal conditions. Microbial virulence as defined has been best studied and most clearly defined experimentally in the case of pathogenic bacteria (Finlay and Falkow, 1997). This is especially true among some extracellular toxigenic pathogens. While they are susceptible to phagocyte-mediated killing, they often cause acute infectious diseases by releasing exotoxins (Arbuthnott, 1978; Lubran, 1988). In many of these cases, the molecular actions of these toxins have been established as the direct cause of the virulent phenotype manifested by the clinical symptoms observed (Middlebrook and Dorland, 1984; Olsnes et al., 1991; Bhakdi, 1998).

However, many chronic infectious diseases are caused by non-exotoxigenic pathogens, which often dwell in tissues or survive and live intracellularly. Among the latter are the etiologic agents of leishmaniasis-trypanosomatid protozoa, which parasitize macrophages as their principal host cells. The virulence of such parasites is manifested in human infection by their ability to produce different clinical symptoms, varying from self-healing cutaneous lesions to potentially fatal visceral disease. These diseases, especially the latter, are marked by disorders of hemato-lymphoid systems, e.g. lymphadenitis, hyperplasia of bone marrow and hepatosplenomegaly, resulting in prominent clinical signs, including fever, cachexia, anemia, pancytopenia, hypergammaglobulenemia and the resultant serum IgG/albumin ratio reversal (Bryceson, 1996). Virulence of these parasites thus appears to be based wholly or in a great part on a pathological response of the host immune system in leishmaniasis. This is not unusual for intracellular pathogens, against which host immunity is often manifested as a 'two-edged sword' of either 'healing' or 'pathology'.

It has been shown previously that patients with leishmaniasis develop an immune response to parasite-unique epitopes, all of which originate unexpectedly from parasite intracellular antigens. These are largely evolutionarily highly conserved cytoplasmic molecules (Mougneau et al., 1995; Requena et al., 2000; Kar et al., 2000; Probst et al., 2001) collectively referred to here as 'pathoantigens'. In contrast, there is little or no immune response in natural infection against *Leishmania* surface or secreted molecules. Indeed, many of them are crucial for these parasites to establish infection of hosts or host cells (Chang and Fong, 1983; Chang et al., 1990; Alexander and Russell, 1992; Bogdan and Rollinghoff, 1998, 1999; Kane and Mosser, 2000; Rittig and Bogdan, 2000). These invasive/evasive determinants are by necessity exposed to the immune system when they infect susceptible hosts. It is thus possible that these determinants may have been evolutionarily selected to become immunologically 'invisible', thereby facilitating leishmanial invasion of hosts by evading their microbicidal immune response.

A hypothetical model is thus proposed to explain *Leishmania* virulence by implicating the two different groups of relevant parasite molecules (Chang, 1993; Chang et al., 1999; Chang and McGwire, 2002). The invasive/evasive determinants of these pathogens themselves do not cause diseases per se, but are necessary for the establishment of infection as a prerequisite for virulence.

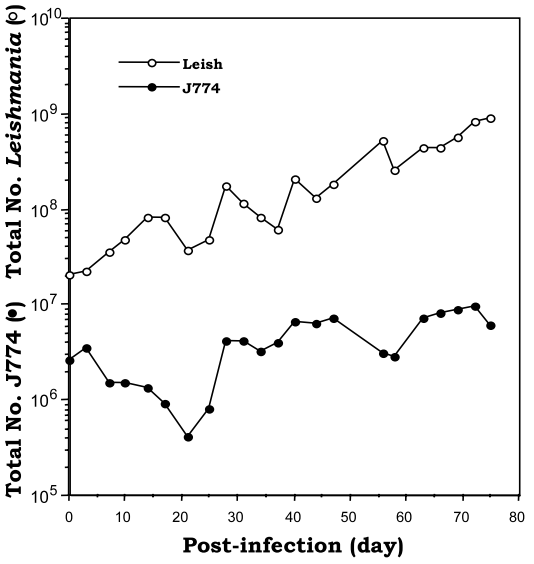

Fig. 1

Virulent phenotype is, in fact, manifested as the clinical immunopathology seen in the disease, resulting directly from the pathoantigenicity of the parasites, i.e. interactions of their pathoantigenic determinants with the host immune system. Virulence is therefore considered in this model to encompass two separate events: infection sustained by parasite replication and immunologically based pathology. Multiple molecules from parasites are envisioned to participate in each of the two events, rendering their interrelationships all the more intricate in considering host–parasite interactions.

Here, laboratory observations are presented to facilitate the discussion of parasite multiplication versus pathoantigenicity relevant to the model proposed. Specifically, we describe the continuous cultivation of *L. amazonensis* in macrophages of the J774 and other monocytic cell lines. We discuss some inherent properties of intracellular amastigotes, which make these host–parasite systems self-sustainable or self-renewable. Parasite replication is thus considered to participate indirectly in *Leishmania* virulence by maintaining the infection, thereby increasing their 'pathoantigenicity'. The latter entails cytolysis of intracellular amastigotes to release cytoplasmic 'pathoantigens', whose interactions with the host immune system causes clinical immunopathology. A hypothetical scheme is presented for immunological recognition and immunopathology of a proposed pathoantigenic B-cell epitope or K39, to which apparently non-

Fig. 1. Macrophages of J774G8 infected with *Leishmania amazonensis* and grown continuously with intracellular amastigotes of this species. Approximately 2×10^6 macrophages were initially infected with 20×10^6 stationary phase promastigotes of *L. amazonensis* (LV78) in a 25 cm^2 TC flask with RPMI Hepes-buffered to pH 7.4 with 20% heat-inactivated fetal bovine serum. Thereafter, infected cultures were incubated at 35 °C with medium renewal every 3–4 days. At the time points indicated, cells were stripped from the bottom in order to count the total number of macrophages, the percent of infected cells and the average number of amastigotes per infected cells. The value for the total number of intracellular amastigotes per culture was estimated from these counts. Summarized below are features of relevance to this in vitro culture system (Chang, 1980; Chang et al., 1986). After infection overnight (= ≈16 h), essentially all parasites are endocytized by the macrophages, with very few extracellular parasites visible. Large parasitophorous vacuoles typically produced by this parasite begin to appear in infected cells at this time and increase in number and size (see Fig. 2A). Differentiation of promastigotes into amastigotes reaches completion in ≈10 days (Fong and Chang, 1981). The total number of parasites per culture (Fig. 1) (Chen et al., 2000) increases invariably from day 3 to day ≈17. During this period, J774G8 cells may gradually decline in number, concomitant with a steady increase of intracellular parasites. The infection eventually becomes uneven, i.e. 20–30% cells infected each with dozens or hundreds of amastigotes. Stagnation or decline of both cell populations is reversible by changing the medium more frequently to increase the growth of non-infected and lightly infected macrophages. The mechanical actions during medium renewal inadvertently break up heavily parasitized cells. The amastigotes so released serve to inoculate non-infected cells, restoring a more even rate of infection, i.e. ≈70% of cells infected with an average of approximately ten amastigotes per cell. This condition favors replication of intracellular amastigotes. The infected culture can be maintained continuously this way by repeating the same maneuvers with cyclic growth of both J774 cells and their intracellular amastigotes. Ultimately, subculture of these infected cells by splitting them periodically into two becomes necessary. Continuous maintenance of these infected cells is interrupted only by inadvertent introduction of adverse conditions due invariably to human error or equipment failure. Untimely medium renewals or subculturing, for example, lead to the degeneration of the infected cultures or over-replication of amastigotes to overwhelm the macrophages. On other occasions, intracellular parasites are cleared from infected cultures under conditions favorable to J774 cells for their rapid growth or unfavorable to the amastigotes for their survival, e.g. a temperature shift-up from 35 to 37 °C or higher (Chang et al., 1986).

protective antibodies are elevated to exceedingly high titers in Indian kala-azar patients.

2. Host-parasite co-existence—slow growth of intracellular amastigotes and their ability to prevent infected macrophages from cytolysis

We have studied *L. amazonensis* amastigotes in J774 lines of mouse macrophages in vitro for over two decades (Chang, 1980; McGwire and Chang, 1994; Chen et al., 2000). Our observations have left us with the impression that *Leishmania* infect these cells and replicate in them, but do not cause their rapid degeneration or lysis. Fig. 1 serves to illustrate this point by showing the growth of both macrophages and amastigotes together in an infected culture (see Fig. 1 for technical details of relevance). We have maintained such infected macrophages for more than 2 years as the longest stretch of continuous cultivation, although the collection of quantitative information was terminated on day 78 for the experiment shown in Fig. 1. We have also obtained similar results with THP-1 cells of a human macrophage line. After infection of these phagocytes in vitro, *L. amazonensis* readily grows intracellularly and produces clearly visible changes in macrophages, as observed in vivo. Some of these observations are consistent with those of macrophages infected by other pathogenic *Leishmania* spp., although different conditions are required (Chang et al., 1986). Where appropriate, comparable in vivo observations will be mentioned on the basis of previous experiences with this species in mouse models and *L. donovani* in hamsters (Chang and Hendricks, 1985). Further discussed below in some details is the intracellular site of parasitization by amastigotes and their properties and activities relevant to their own survival and that of the infected host cells.

All pathogenic *Leishmania* spp. are known to replicate in the modified phagosome-lysosome vacuolar systems of infected macrophages referred to as parasitophorous vacuoles. Most striking is the huge parasitophorous vacuoles produced by infecting macrophages with *L. amazonensis* and related species (Fig. 2A). These vacuoles vary in size and contain amastigotes variable in number, ranging from several to dozens to hundreds. These vacuoles appear to be fluid-filled as if turgor pressure is built up, making them look like 'spheres' inside the macrophages. Not all vacuoles in infected macrophages are so striking, especially in mitotic and heavily parasitized J774 cells (Fig. 2B–D). The appearance of huge parasitophorous vacuoles is associated with in vitro and in vivo infection by living virulent parasites, since they are absent in cells infected with killed parasites or avirulent lines and disappear upon anti-*Leishmania* therapy (Ramazeilles et al., 1990). Amastigotes appear to adhere to the vacuoles (Fig. 2A) with tight abutment of amastigote and vacuolar membranes (Figs. 3 and 4, see section between arrows). Exchanges of molecules presumably occur via this host–parasite interface (Schaible et al., 1999; Henriques and de Souza, 2000). Macrophage MHC Class II and H2-M are recruited to the junction region and eventually enter amastigotes for degradation (Antoine et al., 1999). The immunological and biological significance of these events has been discussed (Antoine et al., 1998; Courret et al., 2002). Whether there is additional ligand-receptor binding for signal transduction of some kind or exchanges of signal or nutritional molecules at this host–parasite interface awaits further investigation. This possibility is suggested by the findings that *Leishmania* infection of macrophages appears to substantially suppress their gene expression, as determined by gene array analysis (Buates and Matlashewski, 2001).

Of particular relevance is the ability of amastigotes to replicate in these distended vacuoles of macrophages without rupturing them. The principal reason is that amastigotes replicate slowly in these parasitophorous vacuoles, since they have a long generation time. Under optimal conditions, both in vitro and in vivo, this is estimated to be $\approx$ 24 h—three times longer than that of axenic amastigotes grown under macrophage-free conditions (Pan et al., 1993). If axenic amastigotes are indeed biologically equivalent to intracellular amastigotes (Bates, 1994), the microenvironment of the parasitophorous vacuoles would seem to constrain the growth of the latter. The vacuolar conditions might impose on amastigotes the con-

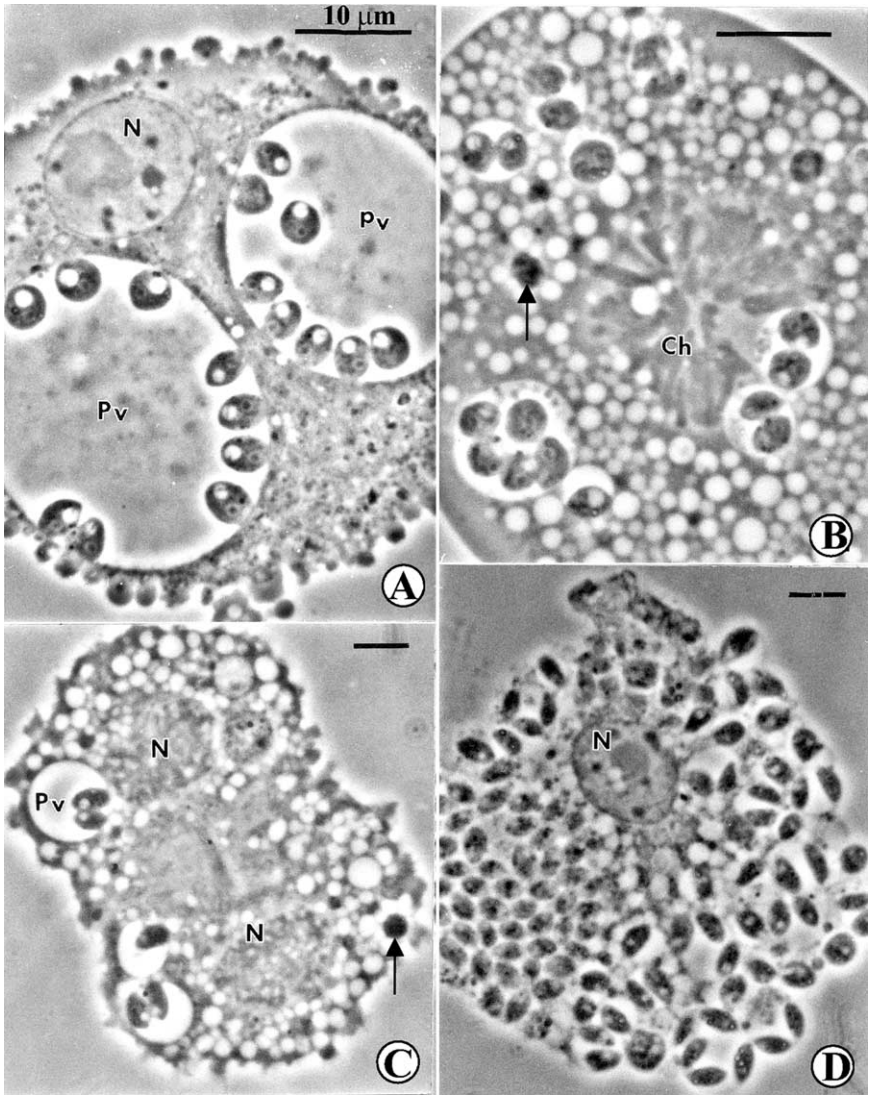

Fig. 2. Appearance of intracellular amastigotes in different parasitophorous vacuoles of J774G8 macrophages after long-term infection with *Leishmania amazonensis.* (A) Typical fluid-filled large vacuoles with amastigotes adhered to the vacuolar membranes; (B, C) amastigotes in smaller vacuoles in J774 cells undergoing mitosis; (D) an intact, but apparently degenerating macrophage infected with >100 amastigotes. Arrows point to apparently degenerating amastigotes. Phase contrast microscopy. Bar = 10 microns.

straints of a limited nutrient supply for essential growth factors, such as heme compounds (Sah et al., 2002) or the building blocks for the biosynthesis of their essential surface molecules, i.e. glycosylated phosphatidylinositols (Mensa-Wilmot et al., 1999). The rate of amastigote replication is comparable to, if not slower than, that of the J774 cells in culture. In any case, the provision of macrophages to *Leishmania* for infection apparently keeps pace with such slow growing amastigotes. This explains the presence of a mixture of non-infected, lightly infected and heavily infected macrophages observed in both in vitro and in vivo settings. New macrophages to serve as host cells for the parasites are provided via replication of non-infected and lightly infected cells in the J774

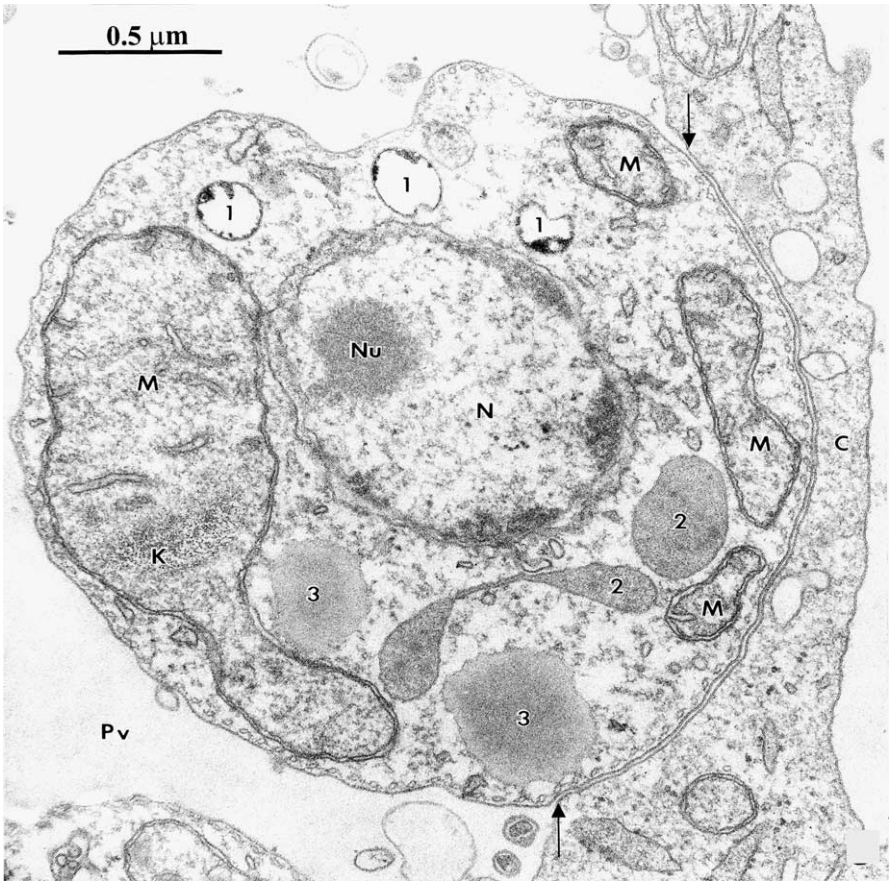

Fig. 3. A transmission electron micrograph showing close apposition of amastigote plasma membrane and parasitophorous vacuolar membrane in an infected J774 cell. Arrows delineate the close abutment of parasite and host membranes. c, Cytosplasm of host cell; k, kinetoplast DNA; M, mitochondria; N, nucleus; Nu, nucleolus; Pv, parasitophorous vacuole; (1) cytoplasmic granule type 1, probably 'acidocalcisome'; (2) cytoplasmic granule type 2, presumably lysosome or megasome; (3) cytoplasmic granule type 3, presumably glycosome. Bar = 0.5 micron.

in vitro system, while those in infected animals are recruited to the lesion site from those in circulation. Irrespective of their origin, it is likely that the emergence of new macrophages for infection, coupled with the slow growth of amastigotes contributes to the varied levels of infection seen. Otherwise, rapid and massive cytolysis of infected cells would be expected.

Amastigotes also appear to act in the interest of self-preservation by allowing infected macrophages to maintain their functional and structural integrity. This seems to vary with parasite loads at different stages of infection. Lightly infected cells are left intact functionally, as indicated by their ability to phagocytize additional amastigotes and undergo mitosis to provide the latter with additional habitats (Fig. 2B, C). However, it has been consistently observed that a small number of intracellular amastigotes become degenerate (Fig. 2B, C; arrows) or undergo cytolysis in parasitophorous vacuoles. This event cannot be interpreted beyond the assumption of spontaneous or inadvertent cell degeneration, as observed in any culture of microorganisms. Interestingly, *Leishmania* have been suggested to undergo programmed cell death via apoptosis (Moreira et al., 1996; Arnoult et al., 2002; Lee et al., 2002). More significantly, there is evidence that heavily infected

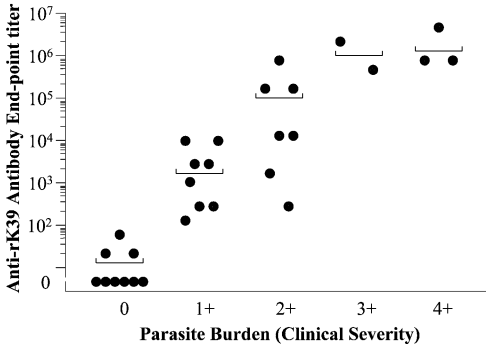

Fig. 4. Positive correlation of anti-K39 antibody titers with parasite burdens in Indian kala-azar patients. See Qu et al. (1994) and Singh et al. (1995) for experimental details for sample collections, end-point titration of anti-K39 antibodies by ELISA and grading for parasite burdens by microscopy.

cells are prevented from premature degeneration, although they must be grossly impaired functionally. Indeed, macrophages have been observed to maintain their integrity both in vitro (Fig. 2D) and in vivo (Soong et al., 1997) even when they carry heavy parasite loads in the hundreds per cell. Experimental evidence indicates that *Leishmania* infection of phagocytes, i.e. macrophages (Moore and Matlashewski, 1994) and neutrophils (Aga et al., 2002), inhibits their lipopolysaccharide-induced apoptosis. This appears to be mediated by enzymes (manuscript in preparation) released along with gp63 from *Leishmania* (McGwire et al., 2002). Perhaps, *Leishmania* may be equipped with additional mechanisms to prevent their host cells from undergoing necrosis and/or apoptosis via different signal pathways.

In summary, the observations described above suggest that *Leishmania* co-exist with macrophages by living in their parasitophorous vacuoles. This is apparently due primarily to the slow growth rate of amastigotes and in part to their ability to preserve the structural, if not functional, integrity of infected cells. The host–parasite co-existence is not a completely peaceful one. The population of heavily infected macrophages builds up slowly. They eventually become degenerated and thus susceptible to phagocytosis by other macrophages, thereby spreading the infection laterally (see below). Intracellular multiplication of *Leishmania* thus appears to serve the aim of maintaining infection after the sequential steps of their entry into these phagocytes and survival in them.

3. Amastigote cytolysis and replication in relation to *Leishmania* virulence

Although *Leishmania* infect macrophages without causing extensive destruction of these phagocytes in vitro, a small number of amastigotes appear to occasionally undergo spontaneous degeneration and cytolysis (see above). This is expected to occur more extensively in diseased hosts, considering the presence of other immune factors recruited to the immediate surrounding of infected cells. Interestingly, infected organs or lesions of susceptible hosts often contain heavily parasitized macrophages, but rarely intact free extracellular amastigotes, even in the active disease phase. Regardless of whether heavily parasitized cells remain intact or partially disintegrated, these cells along with their contents may well be ‘cleared’ or phagocytized by other macrophages long before their amastigotes are set free. This means of spreading amastigotes from one host cell to another is expected to favor their survival by avoiding exposure to the lytic factors present in body fluids. It is thus advantageous for parasites to prolong their intracellular residence by protecting infected cells from rapid cytolysis, accounting for the presence of heavily infected macrophages. There are situations where infected cells are lysed along with the intracellularly located amastigotes. This event is noted only in the necrotic center of the lesion or that of granuloma at later stages of the disease (Terabe et al., 2000). The scenarios presented above underscore the occasions when some amastigotes do undergo cytolysis, despite their apparent activities to prevent premature disintegration of their host cells.

Cytolysis of amastigotes may be a principal way to present some of their intracellular molecules to the host as ‘pathoantigens’. Indeed, essentially all immunologically active epitopes in leishmaniasis characterized so far have been found to originate from evolutionarily conserved cytoplasmic molecules of these parasites. Examples include struc-

tural proteins, cytoplasmic enzymes, chaperonins and proteins in ribosomes, chromatin and glycosomes (Mougneau et al., 1995; Requena et al., 2000; Kar et al., 2000; Probst et al., 2001). Of particular relevance are two additional ones: an RER nuclease of *L. pifanoi* amastigotes (Haberer et al., 1998) and a kinesin-like molecule expressed only in the amastigotes of visceral *Leishmania* spp. (Burns et al., 1993). Cytolysis of amastigotes would seem to be the only plausible explanation for exposure of these epitopes to the host immune system especially if this event proves to be essential in eliciting the initial recognition and/or subsequent immunopathological response. Several 'pathoantigens' have been examined to delineate their immunological epitopes. They are all localized in each specific sequence to a region that is unique to *Leishmania*, but different or absent in the host homologous gene (Burns et al., 1993; Mougneau et al., 1995; Soto et al., 1995a,b). This sequence specificity argues for *Leishmania*-specificity of the immunopathology seen in leishmaniasis, perhaps independent of autoimmunity and polyclonal B cell activation by non-specific antigens.

The release of 'pathoantigens' to cause clinical immunopathology by the manner proposed may paradoxically require parasite replication. This is true, even though the latter event is in itself not directly responsible for the virulent phenotype. As discussed earlier, replication of amastigotes is limited, but crucial for sustaining the infection. In its absence, there would be no parasites available for the release of their 'pathoantigens'. This release is predicted to increase with parasite loads built up via amastigote replication. In that sense, the dosage effect of 'pathoantigens', if relevant, must be positively correlated with the degree of parasite cytolysis, whatever mechanisms may mediate this event, i.e. inadvertent or immune-mediated cytolysis and/or programmed cell death.

4. Pathoantigenicity and immunopathology of T-cell and B-cell epitopes in leishmaniasis

The pathoantigenicity of *Leishmania* T-cell epitopes has long been recognized. Immunological aspects of this have been extensively studied, chiefly using *L. major*-mouse models. Most interesting and significant is the initial finding that progression of this infection into healing or disease-phenotype follows the development of CD4+ cells into either the Th1 or Th2 pathway with the elaboration of different cytokine profiles, e.g. IFN-γ and IL12 versus IL 4 and IL10 (Reiner and Locksley, 1995). There is a substantial body of literature to follow up details of this paradigm experimentally in different models (reviewed in Reed and Scott, 1993; Locksley et al., 1999; Solbach and Laskay, 2000). The dichotomy of T cell development is determined presumably within 5–36 h after needle inoculation of mice with infective promastigotes. Subsequent attention has continued to focus on the cell-mediated immune response. Efforts to identify antigens of relevance uncovered a dominant T-cell epitope in LACK (*Leishmania* homolog of receptors for activated C kinase) (Mougneau et al., 1995). How this and other epitopes trigger T-cell development along the protective Th1 pathway is under intensive investigation. Currently, emphasis is placed on their presentation by dendritic cells (see Solbach and Laskay, 2000; Qi et al., 2001; Moll and Berberich, 2001; Stetson et al., 2002). The drive toward a better understanding of the Th1 pathway is highly significant in the interest of developing much needed effective vaccines against leishmaniasis (Tsikaris et al., 1996; Piedrafita et al., 1999; Handman, 2001; Reed, 2001).

The immunopathological events subsequent to development of the Th2 phenotype have received less attention. Nevertheless, there is an increasing awareness that this is not simply due to uncontrolled amastigote replication. The importance of parasite antigen specificity has also gained increasing recognition (Colmenares et al., 2002), mainly due to observations that different parasite species may produce very different outcomes in the same strain of mice (see Solbach and Laskay, 2000). However, our knowledge in this area remains descriptive. It has been observed that inflammation recruits various immune cells to the original site of infection as well as to secondary sites, in which one finds infected macrophages after metastasis. During the subsequent chronic phase of disease, there is a gradual accumulation of addi-

tional immune cells, largely T- and B-cells. This is especially prominent in draining lymph nodes of cutaneous lesions and in the bone marrow, spleen and liver of kala-azar patients. Undoubtedly, the physical expansion of immune cell populations and immune mediators released from them contribute to the clinical symptoms and signs of leishmaniasis.

The 'pathoantigens' of concern may originate from different pools of parasites, which are known or observed to undergo cytolysis at different times after infection. Those from the original inoculum delivered by a needle or by a sand fly bite may not be totally excluded, since the infective promastigotes therein are susceptible to cytolysis extracellularly by serum lytic factors and intracellularly by professional phagocytes, especially neutrophils and eosinophils (Pearson and Steigbigel, 1981; see Chang and Fong, 1983). In natural infection, these potential 'pathoantigens' must be very small in quantity, considering their origin from only a fraction of several hundreds of promastigotes—the number expected to be delivered by each sand fly bite (see Belkaid et al., 1998). In addition, their release is a transient occurrence long before the onset of any visible clinical symptoms. The possibility nevertheless exists that these promastigote antigens may 'prime' or 'trigger' a specific function and/or play an indirect role by hitherto undefined mechanisms. It is more likely that the major pools of proposed 'pathoantigens' are derived from amastigotes, which are lysed together with their host cells. Alternatively, such 'pathoantigens' may be released from a small number of amastigotes, which are lysed spontaneously within infected cells during the infection. Immunological response to these 'pathoantigens' is suggested by a build-up of immune cells at the sites where infected macrophages are located (or transiently at the sites where parasite antigens are injected, such as in the Leishmanin skin test). Thus, evolution of the virulent phenotype as defined appears to require continuous elaboration of *Leishmania* 'pathoantigens'. This assumption is supported not only by the absence of such immunopathology in non-infected and unrelated organs or tissues, but also its disappearance from these affected sites after chemotherapy. The progressive and sustained release of 'pathoantigens' from lysed amastigotes thus appears necessary for maintaining the virulent phenotype seen.

The proposed 'pathoantigens' are predicted to contain unique *Leishmania* epitopes specific to both T- and B-cells, as suggested by the expansion of both cell types in lymphoid and other organs of concern. Presumably, 'pathoantigens' are processed cytosolically and/or lysosomally by macrophages or dendritic cells for presentation via MHC-class I and/or MHC-class II pathways (Kima et al., 1997). The antigens may be endogenously derived from few lysed amastigotes within infected cells and/or may be acquired exogenously from other disintegrating infected cells. Which of the many T-cell epitopes are necessary and actually presented to trigger and maintain expansion of Th2 cells related to their immunopathology is unclear, although an epitope of LACK is known to elicit a Th2-type response under certain experimental conditions (Stetson et al., 2002). Interestingly, some antigens identified by screening *Leishmania* expression libraries with T-cells from a self-healing subject (Probst et al., 2001) and with patients' sera (Requena et al., 2000) fall within very similar or even identical molecules, e.g. heat-shock proteins, histones and tubulins. The T- and B-cell epitopes of these *Leishmania* molecules await further investigation. Although several *Leishmania* epitope sequences recognized by patients' specific antibodies in kala-azar sera have been defined (Requena et al., 2000), there is a paucity of information as to how *Leishmania*-specific T- and B-cell epitopes, especially the former, are involved in human leishmaniasis.

5. B-cell epitope K39 in kala-azar: a hypothetical consideration of its immunopathology and pathoantigenicity

A potential pathoantigenic epitope may be K39, the 39 amino acid repeats in a kinesin-like molecule present in visceral *Leishmania* spp. and expressed only by their amastigote stage (Burns et al., 1993). Patients with visceral leishmaniasis who have active disease often produce abundant anti-

K39 antibodies (IgG) (Qu et al., 1994; Ozensoy et al., 1998), reaching an especially high titer of 10^{-6} in Indian kala-azar (Singh et al., 1995; Kumar et al., 2001; Singh et al., 2002). There is a positive correlation between the titers of these antibodies and parasite or 'pathoantigen' loads in the bone marrow. The K39 repeats have been subsequently developed for serodiagnosis of kala-azar in dipstick format, which proves to have a high degree of specificity and sensitivity for this disease in Asia and South America (Sundar et al., 1998; Delgado et al., 2001). Immunological response to K39 is thus present in most, if not all, visceral disease, the most virulent form of leishmaniasis. The significance of this is further underscored by the fact that anti-K39 antibodies are absent or present at a low level in cutaneous leishmaniasis (Burns et al., 1993; Ozensoy et al., 1998)—a self-curing, less virulent form of leishmaniasis. With the target antigens inside the amastigotes, anti-K39 antibodies cannot be considered to have any antileishmanial function. On the contrary, their over-production can only contribute to the immunological and hematological disorders of the patients. The presence of these antibodies in such high titers contributes to the IgG/albumin ratio reversal typically seen in kala-azar patients' plasma. Immunoglobulins in such abundance may functionally impair Fc receptor-bearing leukocytes and cause pathological conditions related to the formation of aggregated IgG and immune complexes (Requena et al., 2000). High titers of the antibodies in question further indicate that K39-specific B cells are significantly expanded. That may conceivably contribute to the hyperplasia and thus functional impairment of bone marrow, spleen and other affected organs. It is likely that overall clinical immunopathology seen in kala-azar results from cumulative effects of different epitopes, but not just K39 alone, on the host immune system.

Still, K39 may be considered as a surrogate marker of immunopathological epitopes, if not a major or even a representative one, for visceral *Leishmania* virulence. There are many potential pathways to consider the issue of how K39-specific B-cells may emerge for expansion. Although polyclonal activation by non-specific antigens cannot be totally ruled out, it is considered unlikely due to *Leishmania*-specificity of the K39 sequence. The simplest scenario may be to consider the repetitive nature of K39. As such, it may have modest activities to prime B-cell clones of relevance directly for limited expansion. This expansion may be escalated to a high level in the presence of favorable cytokine environments, such as IL4, IL10 and TGF β (Gomes et al., 2000). These cytokines are indeed abundant in the Th2 disease phenotype that has been shown to develop rapidly within 5-36 h after infection with promastigotes in experimental leishmaniasis (see Solbach and Laskay, 2000). If this holds true in the natural infection for kala-azar, the favorable cytokine environments may already be in place when amastigotes are available to provide kinesin-like molecules as a source of 'pathoantigens' after differentiation from promastigotes. This event is known to take many more days to complete (Fong and Chang, 1981; Doyle et al., 1991; Saar et al., 1998). Little or no anti-K39 antibodies are produced when visceral leishmaniasis is opportunistically associated with AIDS (Alvar et al., 1997). Since these patients are known to have their CD4+ cells depleted, the involvement of these cells in the production of anti-K39 antibodies is apparent. It is difficult to predict the origin of the T-cell epitopes that may be involved in the expansion of K39-specific B-cells in kala-azar. They may be a portion of K39, portion(s) of the kinesin-like molecules, other *Leishmania* antigens or even those of host origin. Whatever mechanism may be involved, K39-specific B-cells must be expanded significantly to account for production of anti-K39 IgG with titers much higher than those against other epitopes. It is possible that lower titers detected for the latter group may be due to their lack of repetitiveness needed for the initial priming of relevant B-cells.

6. Conceptual and practical significance of the proposed model

The proposed model conceptualizes two groups of parasite molecules to explain microbial virulence in leishmaniasis, which is caused by an intracellular pathogen. Parasite surface and secre-

tory molecules, as one group, ease some parasites into their destination quietly and smoothly for growth in the parasitophorous vacuoles of macrophages. Presumably, these molecules have long been evolutionarily selected by exposure to the host immune system for 'invisibility'. This immunological 'invisibility' may be based directly or indirectly on the same principle of mutation-adaptation for the 'best-fit'. Antigenicity thus may be lost directly from the molecules of concern. Or, indirectly, their expression may become substantially down-regulated after completing their initial tasks for infection, as seen with *Leishmania* gp63 and lipophosphoglycans. The other group consists of parasite intracellular molecules, which include those necessary for 'house-keeping' that have not been implicated previously in microbial virulence. As such, they may not have been subjected to host-selective pressure. They are, thus, inherently 'foreign' to the host, but are also 'invisible' by taking intracellular residence wherein they are protected by the first group of molecules. These intracellular molecules of parasites become 'visible' upon cytolysis, making 'pathoantigens' available to cause the virulent phenotype. Polarization of the two groups with respect to their cellular origin is an over-simplification for the convenience of presenting the concept. While the surface and secretory molecules of most parasites may be largely immunologically 'invisible', not all intracellular molecules are antigenically active or provocative in the natural host. In addition, some intracellular molecules are known to help parasite's survival, e.g. a RER Ca-ATPase apparently involved in the development of infective parasites (Rodriguez et al., 2002). Many more cytosolic enzymes are indispensable for the parasite's replication.

Hypothetical separation of parasite molecules into the two groups as discussed may be useful for considering regulation of microbial virulence in general. The model assumes that every pathogen has both infection- and pathology-related molecules as separate functional groups, which interact with the host differently to cause either no disease or a disease phenotype. It is conceivable that inherent properties of the pathogen, coupled with its response to different signals from the external environment, may differentially affect the expression of one or more molecules in either or both groups. Infection of a host by the same pathogen under different conditions may thus produce a spectrum of different outcomes, variable in severity from asymptomatic to disease phenotypes. Although host factors are undoubtedly important in the context of immunity and immunopathology, as discussed, they play a passive role after all in response to the pathogens. Many immune modulators are susceptible to changes due to host heterogeneity. This may occur among individuals with differences in genetic backgrounds, nutritional status, coinfection by other pathogens and even in response to insect vector-derived factors. These heterogeneous host factors must be all considered as crucial variables in assessing the epidemiology of every infectious disease. Their inclusion as a whole is however untenable in experimental evaluation of pathogens for their intrinsic capacity in virulence. This will become feasible when host variables can be experimentally more defined qualitatively and quantitatively with the advances in genomic and proteomic biotechnology.

Although the model presented is based on the observations with *Leishmania*, it may hold true at least conceptually for other pathogens, especially with respect to the paradigm of their infection or replication versus pathology or pathoantigenicity. The respective functional molecules within the two groups may vary in relative importance with different pathogens. Viruses, for example, are known to replicate by exploiting the biosynthetic machinery of host cells. In many cases, they directly cause extensive cytolysis of host cells as the principal mechanism of virulence in the acute phase. Pathoantigenicity of certain viral antigens and/or those derived from lysis of infected cells may cause immunopathology in some viral diseases. This may be true even for extracellular pathogens, which are phagocyte-resistant and possess disease-producing exotoxins. Many such bacteria may not cause diseases until they are fortuitously allowed to enter the blood stream or other tissues where they release toxins as they replicate. Replication of these bacteria is clearly important, enabling them to sustain the infection.

Nevertheless, toxigenic bacteria minus their exotoxins may not be totally avirulent, as they are still expected to establish infection. Their cytolysis during infection may release other cellular components to evoke the host pathological response. This outcome is far more likely to occur for some tissue-dwelling extracellular pathogens. The best examples within this group are perhaps those which have evolved unusual mechanisms to defy host humoral immune attacks by changing their surface molecules genetically via 'antigenic variations', e.g. the African trypanosomes (Barry and McCulloch, 2001). Conceivably, this confrontational and suicidal strategy is theoretically favorable to the development of immunopathology, since it periodically produces waves of antibody-lysed parasites that may serve as a potential source of 'pathoantigens'. The *Leishmania* model is perhaps best suited to explain virulence in chronic infectious diseases caused by other intracellular slow growing microbes, for example, in mycobacterial and rickettsial diseases. There are indeed many outward similarities of immunopathology between leishmaniasis and these diseases, although the mechanisms of pathoantigenicity may be expected to differ significantly in fine detail, hence their clinical manifestations. Curiously, most parallel mechanistically to the proposed model in principle, but with considerable exaggerations, are helminth diseases, such as schistosomiasis (Cheever et al., 2000). In this case, non-replicative adult worms reside in the blood vessel and are completely 'invisible' to the host immune system, although they release thousands of eggs daily into the bloodstream. The antigens released from the eggs produce immunopathology, accounting for all the clinical symptoms of the disease. The immunologically 'invisible' and 'visible' components of *Schistosoma* are present in separate entities, but they are merged into the same cell in the case of *Leishmania*. Confronting the host immune system are thus 'pathoantigens' from an army of offspring soldiers dispatched by the formers, but from the latter directly after committing a suicidal mission. The confrontation in both cases ends in the manifestation of virulent phenotype. Here, evolution of parasitism appears to converge into a common theme, despite marked differences between these two groups of parasites in their phylogeny and their niche in the mammalian host.

The conceptual grouping of parasite molecules for virulence as discussed may bear on the strategy of considering attenuation of virulence by genetic approaches (Beverley and Turco, 1998). Molecular attenuation of *Leishmania* virulence might be achieved by genetic modification or elimination of pathoantigenicity (Chang et al., 1999; Chang and McGwire, 2002). The feasibility of this consideration awaits further evaluation of the number and substitutability of genes encoding 'pathoantigens' and the importance of the relevant epitopes in the normal functions of these molecules in *Leishmania*. Negative selection for these 'pathoantigens' based on the strategies of gene or transcript disruption may prove difficult, considering their multiplicity and functional importance in *Leishmania*. The possibility exists that the bulk of immunopathology may be caused by a handful of specific 'pathoantigens' with replaceable offending epitopes. If so, it is technically possible to obtain mutants with significantly attenuated virulence by modifications and replacement of relevant genes. These mutants with intact invasive/evasive determinants are expected to infect the host as usual. If the infection produces a limited virulent phenotype, evidence is provided in support of the model proposed. If the infection is self-limiting, as expected and confers protection against challenges with the virulent wildtype, they suggest the potential use of these mutants as vaccines. This may represent an improvement of 'Leishmanization' where individuals receive live virulent parasites in order to acquire life-long immunity against cutaneous leishmaniasis. In the absence of pathoantigenicity, mutants under discussion may have the dual advantages of producing not only limited or no disease, but also a protective phenotype. This proposal is consistent with the current view of immunoregulation in experimental leishmaniasis; namely, these phenotypes segregate with the dichotomy of CD4+ T-cell development along the Th1 or Th2 pathway and this is regulated by *Leishmania* antigen-specificity. If true, the pathoantigenicity-minus mutants would be left with a collection of 'natural vaccines' to confer a

prophylactic function on the host as in 'Leishmanization', but without its drawback of immunopathology. The presence of 'natural vaccines' in such mutants is supported by recent successes in experimental vaccination using a mixture of *Leishmania* recombinant antigens (Aebischer et al., 2000; Campos-Neto et al., 2001; Ghosh et al., 2001; Coler et al., 2002) and knockout mutants of other *Leishmania* genes (Titus et al., 1995; Alexander et al., 1998; Papadopoulou et al., 2002).

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