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Trypanosomatids: Odd Organisms, Devastating Diseases

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Abstract: Trypanosomatids cause many diseases in and on animals (including humans) and plants. Altogether, about 37 million people are infected with *Trypanosoma brucei* (African sleeping sickness), *Trypanosoma cruzi* (Chagas disease) and *Leishmania* species (distinct forms of leishmaniasis worldwide). The class Kinetoplastea is divided into the subclasses Prokinetoplastina (order Prokinetoplastida) and Metakinetoplastina (orders Eubodonida, Parabodonida, Neobodonida and Trypanosomatida) [1,2]. The Prokinetoplastida, Eubodonida, Parabodonida and Neobodonida can be free-living, commensalic or parasitic; however, all members of theTrypanosomatida are parasitic. Although they seem like typical protists under the microscope the kinetoplastids have some unique features. In this review we will give an overview of the family Trypanosomatidae, with particular emphasis on some of its "peculiarities" (a single ramified mitochondrion; unusual mitochondrial DNA, the kinetoplast; a complex form of mitochondrial RNA editing; transcription of all protein-encoding genes polycistronically; *trans*-splicing of all mRNA transcripts; the glycolytic pathway within glycosomes; *T. brucei* variable surface glycoproteins and *T. cruzi* ability to escape from the phagocytic vacuoles), as well as the major diseases caused by members of this family. However, the present review does not cover all trypanosomatids; for example, the insect trypanosomatids are underrepresented here. On the other hand, reviews on this particular group of parasites have been written by experts in the field [3-12].

Keywords: Trypanosomatids, *Trypanosoma, Leishmania, Phytomonas, Herpetomonas*.

INTRODUCTION

Trypanosomatids are evolutionarily extremely successful, not only because they are found over nearly the entire planet and parasitize all groups of vertebrates, several species of invertebrates (including many insects) and even plants [13-16], but also because there is sound evidence that their ancestors date back to about 100 million years ago [2,17,18]. Although there are roughly a thousand described monoxenous trypanosomatids from insects, it is expected that hundreds of thousands of insect trypanosomatids will eventually be described, especially because new lines of evidence indicate that insects are the ancestral hosts for these parasites [2,5,15,19,20].

The nine genera of trypanosomatids recognized to date (the monoxenous *Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Wallaceina* and *Leptomonas*, as well as the heteroxenous *Trypanosoma*, *Leishmania*, *Endotrypanum* and *Phytomonas*) were classified mostly by morphological features in conjunction with host relationships. The morphotypes that characterize individual genera are cell shape, dimensions and the position of the complex kinetoplast-flagellar pocket relative to the nucleus [15,21] (Fig. **1**). These morphotypes can be altered considerably by the environment, *in vitro* cultivation or the addition of drugs [15,19,22-24].

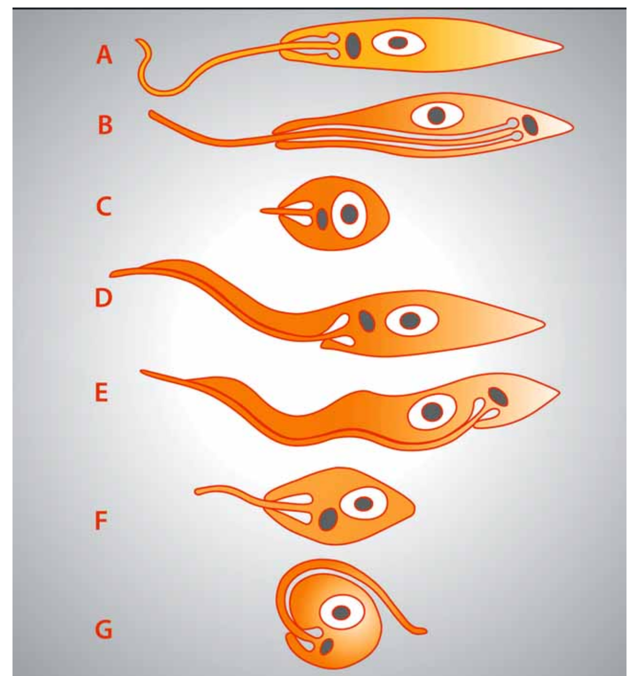

Fig. (1). The most common morphotypes of trypanosomatids. **A**: promastigote; **B**: opisthomastigote; **C**: amastigote; **D**: epimastigote; **E**: trypomastigote; **F**: choanomastigote; G: spheromastigote. Diagram based on Hoare and Wallace [21].

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Biochemical, nutritional and ultrastructural characteristics were added to the classical morphological traits to distinguish trypanosomatid species [25]. Such biochemical features include the complement of cell surface polysaccharides, sialidases and proteolytic enzymes and the content and number of acidocalcisomes. These characteristics, either alone or in combination, are sufficient to distinguish between strains and species, but they usually fail to distinguish between genera [15,26-29]. However, molecular markers involving highly conserved gene sequences (such as small subunit rRNA, 5S rRNA, spliced-leader RNA or a few protein-coding genes) are especially useful for distinguishing between genera [2,15,20,30-32].

Some developmental stages of trypanosomatid protozoan parasites are well suited to cultivation *in vitro*; one such stage is the proliferative stage found in the gut of invertebrate hosts [33]. The most common of these stages is the promastigote, which is the insect stage of *Leishmania* species and the insect trypanosomatid morphotype that grows best *in vitro* (Fig. **1A**). An exception is *Crithidia* spp., whose most commonly observed stage is the choanomastigote (Fig. **1F**) [34]. With respect to African trypanosomes, the slender stage (proliferative in the bloodstream of mammalian hosts) and the procyclic form (proliferative in the fly midgut) grow well *in vitro* and have a trypomastigote shape (Fig. **1E**) [35]. Most studies of *T. cruzi* were performed using the epimastigote form (Fig. **1D**), which is the proliferative stage in the insect host [36]. The life cycles of *T. cruzi*, *T. brucei*, *Leishmania* spp. and *Phytomonas* spp. are discussed later in this review.

The most significant morphological and physiological aspects of trypanosomatids are briefly described here. We divide these aspects into two groups: "common" characteristics, which are traits that are present in other groups of organisms yet are somewhat unique; and "peculiar" features, which are characteristics that thus far have been found only among the kinetoplastids [37]. It is noteworthy that many "unique" biological phenomena first discovered in trypanosomes are in fact more widespread, but cryptic, in other eukaryotes. Examples of this include *trans*-splicing of nuclear RNAs, glycosylphosphatidylinositol (GPI)-anchored membrane proteins and RNA editing. All were first described in trypanosomatids but were subsequently shown to be general traits of eukaryotes [38]. Unless otherwise noted, all of these features will be described herein using *T. cruzi* as a model, as shown in the diagram of the fine structure of the epimastigote form (Fig. **2**) [39].

THE "COMMON" CHARACTERISTICS

Cell Surface

The cytoplasmic membrane with its constituents is the interface between protozoa and their environment. The cell membrane of all examined trypanosomatids is similar to that of other eukaryotes, except for the fact that there are subpellicular microtubules firmly attached to the inner surface of the membrane. The cell surfaces of all trypanosomatids are coated with GPI-anchored proteins and/or free GPI glycolipids, both of which form protective surface layers and/or mediate crucial host-parasite interactions [40,41]. Intracellular trypanosomatids, such as *T. cruzi* and *Leishmania*, initially interact with cell surfaces in vertebrate hosts and then with the membrane of the parasitophorous vacuole. Trypanosomatids also interact with the epithelial intestinal cells of invertebrate hosts [42-44].

Membrane Transporters

Membrane transporters are proteins that possess many alpha helical transmembrane segments and mediate the translocation of various compounds through biological membranes. Some molecules that are small and relatively non-

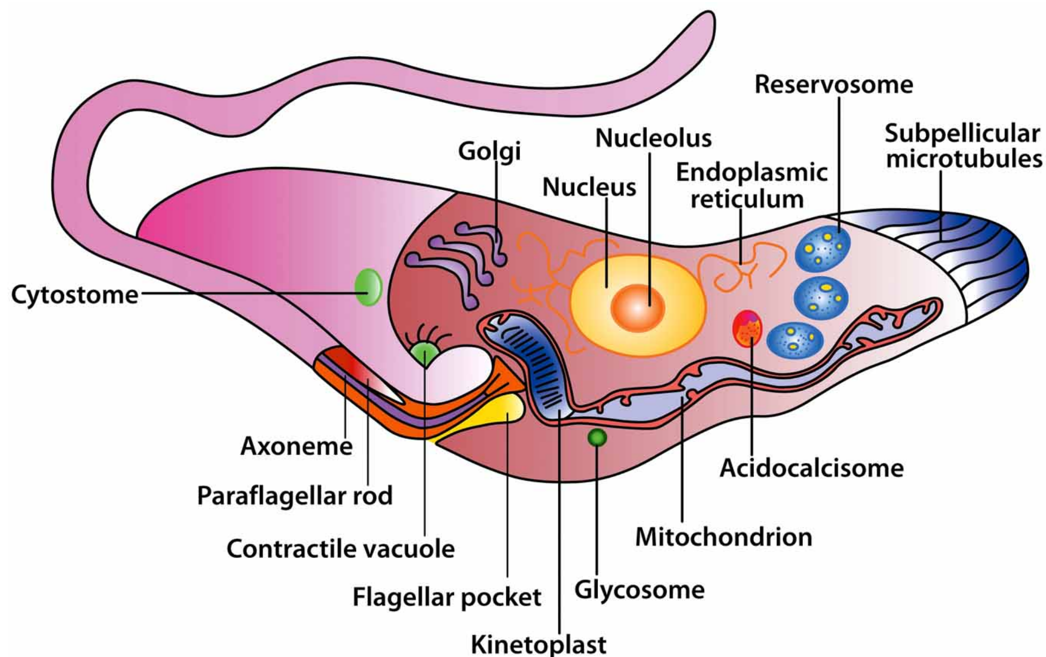

Fig. (2). Main structures and organelles found in the epimastigote form of *Trypanosoma cruzi*. The structures and organelles are depicted as detected in thin sections by transmission electron microscopy. Diagram adapted from De Souza [39] with permission from the author.

polar, such as oxygen, nitrogen and carbon dioxide, can pass through lipid bilayers. All other molecules are too large and/or hydrophilic to diffuse across membranes, so they need transporters to facilitate their passage [45].

Membrane transporters perform a large number of tasks, such as taking up nutrients, expelling metabolites, establishing ion gradients, translocating compounds from one intracellular compartment to another and taking up or exporting drugs [46,47]. Not surprisingly, about 2 to 2.5% of the proteins encoded by the genome of trypanosomatids have been annotated as membrane transporters; some of those are developmentally regulated so that the parasites can cope with severe changes in their environment during the course of their life cycle [45,48,49].

Flagellum

The flagellum is a motility organelle that moves the parasite forward by wave-like beats of the microtubule-based flagellar axoneme [50,51]. All of the stages of trypanosomatids possess one flagellum per cell, even the amastigote form. The flagellum emerges from a basal body in the cytoplasm through a prominent invagination of the plasma membrane called the flagellar pocket [50,51]. Along the length of the cell, the flagellum and cell body are held in close apposition by a network of cytoskeletal and membranous connections that collectively make up the flagellum attachment zone (FAZ) [52]. The flagellum has a typical array of one central microtubule pair and nine peripherally arranged microtubule doublets [53]. A fibrillar structure within the flagellum known as a paraflagellar or paraxial rod is a peculiar structure in the flagellum of trypanosomatids; this rod is made of a complex matrix of filaments connected to the axoneme and is essential for the parasite's survival [53-57]. The flagellar membrane, the flagellar pocket and the pellicular plasma membrane are morphologically, chemically and functionally distinct and have highly diverse biological functions; nonetheless, these three membranes are physically connected and all comprise part of the plasma membrane [50].

In addition to the importance of the flagellum for parasite motility, it is also involved in host-parasite interactions, cell morphogenesis, cell division and evasion from the host-immune system [52]. Pathogenic features of sleeping sickness, Chagas disease and leishmaniasis are directly linked to the migration and binding of the parasites to specific host tissues, which is decisive for disease progression [52,58-60]. Moreover, the flagellum is essential for these parasites to complete each respective life cycle within the insect host, both for traveling and attaching to target tissues [52,61,62].

Intercellular signaling cross-talk between trypanosomatids and their hosts directs the movements and developmental transformations of the parasite within specific host compartments [52], both in mammalian [57,63,64] and insect hosts [52,65-67]. Recent studies have localized cyclic nucleotide and calcium signaling pathways to the flagellum [52,68-71], suggesting that the flagellum provides a signaling platform for environmental sensing [52]. Intracellular signaling cross-talk between the flagellum and several organelles and the structures to which it is connected, e.g., the FAZ, the flagellar pocket, the kinetoplast and the mitochondrion, directs their arrangement and organizes them for segregation during cytokinesis [72].

The flagellar pocket is the exclusive site of endocytosis and secretion in trypanosomatids [73] and it has long been speculated that flagellar beating influences entry into the flagellar pocket [52]. Flagellar motility mutants of bloodstream-form *T. brucei* are not able to engulf VSG complexed with immunoglobulin (Ig) [74]. As clearance of VSG-Ig protein complexes from the parasite surface is crucial for avoiding destruction by the host immune system, this result strongly suggests that flagellar motility contributes to immune evasion and persistent infection [74].

Cytoskeleton

In all trypanosomatids, subpellicular microtubules are distributed throughout the protozoan body, except in the flagellar pocket region. The subpellicular corset consists of microtubules that are connected to one another and to the plasma membrane. The microtubules form a helical pattern along the axis of the cell underlying the plasma membrane together with a regularly spaced intermicrotubule [39,75,76].

Microfilaments have never been observed in the cytoplasm of *T. cruzi*. On the other hand, biochemical and genomic analyses identified a potential role for an actin-myosin system in *T. cruzi*, as this protozoan possesses an expanded myosin family and a CapZ F-actin capping complex in addition to an actin gene [77-79].

Acidocalcisomes

Acidocalcisomes are acidic calcium-storage organelles that were first described in *T. brucei* and *T.cruzi* [80,81]. Later, acidocalcisomes were also described in the protozoan parasites *Toxoplasma gondii* [82] and *Plasmodium* spp. [83], in the green alga *Chlamydomonas reiinhardtii* [84], in the slime mold *Dictyostelium discoideum* [85], in bacteria [86,87] and in human platelets [88]. Acidocalcisomes were morphologically identified by light microscopy more than one hundred years ago as a different kind of granule [89]. These organelles are observed in Giemsa-stained preparations and with the use of dyes that accumulate in acidic compartments [84-88,90].

The morphology and the number of acidocalcisomes vary from species to species and among the developmental stages of the same species [90,91]. By transmission electron microscopy (TEM), *T. cruzi* acidocalcisomes appear as round electron-dense structures with an average diameter of 0.2 µm and their distribution varies among the three developmental stages of the parasite. X-ray microanalysis, ^{31}P-NMR and biochemical techniques indicate the presence of Na, Mg, S, Cl, K, Ca, Zn, O, Fe and P (the latter concentrated in the form of inorganic phosphate and as short-chain polyphosphate (poly P)) [92]; amino acids [93]; and various enzymes [84,94]. The membrane of *T. cruzi* acidocalcisomes contains a specific glycoinositolphospholipid (GIPL) and low concentrations of 3β-hydroxyesterol and as observed in other organisms, a vacuolar ATPase (V-H^+ATPase) and a vacuolar pyrophosphatase (V-H^+PPase) that establish the H^+ gradient; transporters (for basic amino acids, phosphate (Pi), pyrophosphate (PPi) and ions such as Ca (Ca^{2+}-ATPase), Mg, Zn and Fe); exchangers (Na^+-H^+-exchanger; Ca^{2+}-H^+-

exchanger); and channels (a Cl^- channel and a water channel or aquaporin) [95].

The different functions attributed to acidocalcisomes include: phosphorous storage (which may be involved in environmental stress response, differentiation [96] and/or osmoregulation [88,97,98]); the storage of cations (mainly calcium, which is related to the host cell invasion process [99,100]); and pH homeostasis due to the generation of H^+ from the hydrolysis of polyphosphate. Due to the importance of acidocalcisomes in trypanosomatid metabolism and their unique features observed in different organisms, this organelle is considered an important target for the development of new drugs [101,102].

Nucleus

The nucleus is enveloped by a typical porous membrane, which encloses a nucleolus and condensed chromatin dispersed throughout the nucleoplasm [103-105]. During the division process, the nuclear membrane remains intact, intranuclear microtubules appear, the chromatin disperses and dense plates appear, whose number varies depending on the trypanosomatid species [104]. Still, there is no evidence that these plates correspond to chromosomes, which have thus far been detected only using biochemical/molecular biological approaches [106].

A combination of scaffolds, synteny maps and end sequences from *T. cruzi* bacterial artificial chromosome (BAC) libraries was used to organize the majority of *T. cruzi* contigs into chromosome-size assemblies [107,108]. Additionally, a set of chromosomes was validated experimentally via Southern blot analysis using individual genes as probes to confirm the predicted organization of each chromosome. Using this approach, 41 pairs of chromosomes were assembled; this number is in agreement with the predicted chromosome number based upon pulse-field gel analysis [108].

Endosymbionts

Bacterial endosymbionts live in various plants, animals and protozoa and are especially relevant because they likely represent a transitional stage between bacteria and organelles such as mitochondria and chloroplasts [109]. Bacterial endosymbionts have been observed in some insect trypanosomatids of the genera *Crithidia*, *Blastocrithidia* and *Herpetomonas* [109].

Trypanosomatid endosymbionts possess two membranes; one corresponds to the plasma membrane, which is in contact with the bacterial matrix and the other is an outer membrane that faces the host protozoan cytoplasm [3]. The symbiont matrix contains ribosomes, filamentous genetic material, proteins, enzymes and metabolic intermediates that supplement essential biosynthetic pathways of the host protozoan, such as heme, purines, numerous amino acids and vitamins [11,109]. Recently, Motta *et al.* [110] found that during *Crithidia deanei* cell division the endosymbiont replicates before the basal body and kinetoplast segregations and that the nucleus is the last organelle to divide, before cytokinesis. Taking into consideration that the endosymbiosis in trypanosomatids is a mutualistic relationship, which resembles organelle acquisition during evolution, these findings can be considered an excellent model for the understanding of mechanisms related to the establishment of organelles in eukaryotic cells [110].

THE “PECULIAR” FEATURES

Glycosomes

Trypanosomatids harbor numerous genes sharing apparent common ancestry with plants and/or bacteria [111,112]. Many products of these horizontally acquired genes now function in glycosomes, which are found only in members of the class Kinetoplastea. Glycosomes are spherical structures with a protein-dense matrix surrounded by a single phospholipid bilayer and containing no DNA [114,115]. Although glycosomes are evolutionarily related to the peroxisomes of higher eukaryotes and the glyoxysomes of plants [114,115], glycosomes are distinct from peroxisomes because they harbor the glycolytic pathway, which is the mechanism that allows the conversion of glucose into pyruvate, which in trypanosomatids is more efficient than in most other eukaryotes [14]. Ardelli *et al.* [113] have also identified glycosomes and the glycolytic enzymes hexokinase, fructose-1,6-biphosphate aldolase, triosephosphate isomerase, glucosephosphate isomerase and glyceraldehyde-3-phosphate-dehydrogenase associated with this organelle in pathogenic and nonpathogenic strains of the bodonid *Cryptobia salmositica.* The trypanosomatid glycosomal proteome of *L. major*, *T. brucei* and *T. cruzi* confirmed that glycosomes contain most of the glycolytic enzymes [116-118]. The other divergent feature of trypanosomatid glycosomes is the absence of catalase, a characteristic peroxisomal enzyme [119]. In the pathogenic haemoflagellate bodonid *C. salmositica*, however, catalase is found in the glycosomes [113]. On the other hand, the presence of the enzymes necessary for the initial steps in the production of phosphoglycerate from glucose or glycerol in the glycosomes is shared among trypanosomatids [91,115,119,120] and the bodonid *C. salmositica* [113], which contrasts with other eukaryotes, in which this pathway occurs in the cytoplasm.

Glycosomes, the cytosol and the mitochondrion cooperate in the energy metabolism of kinetoplastids. The insect stages of all human-pathogenic trypanosomatids (*T. brucei, T. cruzi* and *Leishmania* spp.) seem to be capable of metabolizing both amino acids and sugars for their free energy supply; they have a large repertoire of enzymes for carbohydrate metabolism, including the glycolytic pathway and a well-developed mitochondrion with a respiratory chain-linked system for oxidative phosphorylation [121-124]. Glycosomes also have other functions related to the biosynthesis of pyrimidines, purine salvage pathways and the synthesis of ether lipids and β-oxidation of fatty acids [14,119,125]. Amastigote forms of *Leishmania* spp. and *T. cruzi*, as well as insect-stage parasites (called the procyclic form in *T. brucei,* the epimastigote form in *T. cruzi* and the promastigote form in *Leishmania*), have a more elaborate energy- and carbohydrate-metabolic network. In these cells, other ATP-dependent kinases (e.g., phosphoenolpyruvate carboxykinase and pyruvate phosphate dikinase) may be found in the glycosomes, whereas phosphoglycerate kinase (PGK) is relocated to the cytosol [125]. Glycosomes seem to be crucial organelles for the bloodstream form of *Trypanosoma brucei*, as this form of the parasite is exclusively dependent on glycolysis for ATP generation [124].

Because of the absence of DNA and protein translational machinery in glycosomes, glycosomal proteins are encoded in the nucleus, synthesized by cytosolic ribosomes and then imported into glycosomes [120]. Thus, glycosomal proteins need to be tagged by post-translational modifications in order to reach and bind to their target glycosomal receptors [126].

Mitochondria

Mitochondria harbor systems for energy production through oxidative phosphorylation, synthesize key metabolites and iron-sulfur clusters and can be the reservoir for factors that amplify signals for programmed cell death [39,106]. It is worth mentioning that in order to synthesize mitochondrial proteins, all the mitochondrial tRNAs of trypanosomatids are imported from the cytoplasm [127-129]. In cells of multicellular organisms, the number of mitochondria is variable but often quite large. The ultrastructure of mitochondria in protists is usually peculiar. In addition to particularities in the density of the matrix and the number of cristae, individuals in the phylum Apicomplexa and in the order Trypanosomatida have a single ramified mitochondrion [9,39,130] (Fig. **2**). In trypanosomatids, separation of the replicated mitochondrial DNA is directly linked to the segregation of the flagellar basal body and mitochondrial division seems to be a checkpoint for cytokinesis [38,131].

In the order Trypanosomatida, the mitochondria are distributed in branches under the subpellicular microtubules and are dilated at regions in which kinetoplast DNA (kDNA) is present. The volume of each mitochondrion depends on environmental and nutritional resources [132,133]. In *Trypanosoma brucei*, mitochondrial volume and activity are higher in parasites in the midgut and proventriculus where glucose levels are low but are lower in metacyclic salivary gland forms. They reach their lowest values in slender forms from blood, where glucose levels are high and thus favor direct glycolysis [134,135]. In contrast, the relative volume occupied by glycosomes varies in the opposite manner. This volumetric counterbalance has been observed in all genera analyzed so far [136,137].

There are two terminal oxidases in the mitochondrial electron transport chain. One is the usual cytochrome oxidase, which is a cyanide-sensitive oxidase and the other is a cytochrome-independent, salicylhydroxamic acid-sensitive alternative oxidase [9,138-140]. The bloodstream form of *T. brucei* uses glucose as its energy source and suppresses many mitochondrial activities. The bloodstream-form mitochondria lack cytochromes, so that respiration in this form is exclusively dependent on the cytochrome-independent trypanosome alternative oxidase (TAO) [138,141]. On the other hand, the procyclic form that lives in the insect's midgut have a well-developed mitochondrion with a fully functional cytochrome-dependent respiratory system and a reduced level of TAO. The procyclic-form mitochondria produce ATP by both oxidative and substrate-level phosphorylation [138,142]. On the other hand, bloodstream-form mitochondria do not produce ATP but hydrolyze ATP to maintain the inner membrane potential, analogous to the reactions that happen in the plant mitochondrial system [138,143-146].

In higher eukaryotes, the electron-transfer chain is a functional sequence of four major multi-subunit complexes that are randomly dispersed in the inner mitochondrial membrane and designated NADH-coenzyme Q reductase (complex I), succinate-CoQ reductase (complex II), ubiquinolcytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV). The enzyme complexes are connected by two mobile redox-active molecules: ubiquinone and cytochrome *c* [147,148]. In *T. brucei* procyclic forms, ubiquinone can carry electrons from succinate dehydrogenase (complex II) either to the cytochrome-mediated respiratory chain (involving cytochrome *c* reductase (complex III), cytochrome *c* and cytochrome *c* oxidase (complex IV)) or to TAO [142,148]. On the other hand, ubiquinone can potentially carry electrons from NADH:ubiquinone oxidoreductase (complex I) and alternative NADH dehydrogenases either to complex III, complex IV or TAO [149-151]. Although a multisubunit complex I has recently been characterized in *Phytomonas serpens*, the presence of a typical complex I in *T. brucei* is controversial [149]. Hypothetically, complex I may be smaller and highly divergent from its equivalents in higher eukaryotes, or *T. brucei* may have bypassed the need for complex I by using an NADH-fumarate reductase to oxidize mitochondrial NADH, thereby producing succinate as a substrate for complex II [149,152].

Most of the trypanosomatid mitochondrial proteins are synthesized in free cytoplasmic ribosomes and then imported into the organelle [38]. Many other proteins are synthesized within the mitochondria, but as trypanosomatid mitochondrial genomes have lost all tRNA genes, they need to import the entire set of mitochondrial tRNAs [127]. All mitochondrial tRNAs in trypanosomatids derive from eukaryotic-type cytosolic tRNAs that need to function in the context of the bacterial-type translation system of mitochondria [127-129]. In *Leishmania tropica*, the RNA import complex (RIC) induces the transport of tRNAs across natural and artificial membranes [153]. RIC is a multi-subunit protein complex from the mitochondria that can also act as an efficient delivery vehicle for tRNA and other small RNAs into mitochondria within intact mammalian cells [153].

In trypanosomatids, mitochondrial tRNAs and their cytosolic correspondents originate from the same nuclear genes. However, due to compartment-specific post-transcriptional nucleotide modifications, cytosolic and imported mitochondrial tRNAs are often physically different [127]. The tRNAs of trypanosomatids are subject to extensive mitochondrion-specific modifications, which include methylation, thiolation and C to U editing [31,127,154,155]. Using inducible expression of a tagged $tRNA^{Glu}$, Bruske *et al.* [127] showed that it is mainly the thiolated form that is imported to the mitochondrion *in vivo*. Unexpectedly, the imported tRNA becomes dethiolated after import, which explains why the non-thiolated form is enriched in mitochondria [127].

Unusual Mitochondrial DNA: The Kinetoplast

In the majority of trypanosomatids, the kinetoplast forms a disk-like structure; in contrast, trypomastigotes of *T. cruzi* and endosymbiont-bearing trypanosomatids possess a more rounded kinetoplast [39].

Kinetoplast DNA (kDNA) differs from nuclear DNA in its buoyant density, base ratio and degree of renaturation. Moreover, unlike any other DNA in nature, the kDNA of

trypanosomatids is composed of circular molecules that are topologically relaxed and interlocked to form a single network. There are two types of DNA rings, minicircles and the maxicircles. There are several thousand minicircles, which range in size from about 0.5 to 10.0 kb and a few dozen maxicircles, which usually vary between 20 and 40 kb [156-158]. The maxicircles are analogous to the mitochondrial DNA of higher eukaryotes and they encode two rRNAs and several subunits of the respiratory complexes as well as some guide RNAs (gRNAs). The minicircles encode gRNAs that modify maxicircle transcripts by extensive uridylate insertion or deletion in a process known as RNA editing [9,158].

Kinetoplastid RNA Editing

Two different RNA editing systems have been described in the kinetoplast-mitochondrion of trypanosomatid protists. The first is a unique form of post-transcriptional RNA processing that occurs only in the mitochondria of kinetoplastid protists. This editing system involves the precise insertion and deletion of uridine residues mostly within the coding regions of maxicircle-encoded mRNAs to produce recognizable open reading frames (ORFs). This editing system is mediated by short overlapping complementary gRNAs that provide the information for U insertion and deletion on the edited mRNA [159,160] through a series of enzymatic cleavage-ligation steps [9,161].

The second RNA editing system is based upon another derived feature of the kinetoplastid mitochondrial genome, which is the complete lack of tRNA genes and the importation of all mitochondrial tRNAs from the cytosol [4]. Thus, the second system involves editing by a C34-to-U34 modification within the anticodon of imported tRNATrp, thereby permitting UGA stop codons to be read as tryptophan [9].

Trans-Splicing of all mRNA Transcripts

Unlike the majority of eukaryotic organisms, trypanosomatids transcribe all protein-encoding genes polycistronically [162-164]. Most trypanosomatid chromosomes contain at least two polycistronic gene clusters (PGCs), which can be transcribed towards the telomeres or away from the telomeres. Genes from a polycistronic unit in trypanosomatids usually do not code for functionally related proteins [165], which is completely different from how operons function in bacteria and nematodes [162].

Trypanosomatid parasites utilize RNA splicing for the maturation of nuclear pre-mRNA in two distinct ways: *cis*-splicing and *trans*-splicing. Although *cis*-splicing has been observed in *T. brucei* [166], intron removal appears to be a rare event in trypanosomatids, as the trypanosomatid genome sequences have identified only three putative intron-containing genes [167]. *Trans*-splicing proceeds through a two-step transesterification reaction, analogous to *cis*-splicing but forming a Y-shaped structure instead of a lariat intermediate [168,169]. Maturation to translatable monocistronic units in trypanosomatids requires resolution of each coding region by *trans* splicing of a 39-nucleotide (nt) spliced leader (SL) exon and 3'-end polyadenylation [168-170]. The source of the SL sequence was found to be a small capped RNA, the SL RNA [169,171,172]. Thus, the addition of the SL sequence serves two purposes: it functions together with polyadenylation to dismember the polycistronic transcripts and it provides a cap for the mRNA [169]. The SL RNA is involved in the maturation of each and every nuclear mRNA, accounting for approximately 7% of total RNA synthesis [170,173,174]. Rapid substrate SL consumption suggests a dynamic processing mechanism [170]. Substrate SL RNA is modified by eight methylations of the 5-nt cap structure and pseudouridylation at nt 28 (ψ_{28}) [170]. Along with those of the m^7G (cap 0), the methylations of the kinetoplastid cap 4 are the most extensive, with 2'-*O*-ribose methylation of the first four nucleotides and additional base methylations on the first (m_2^6A) and fourth (m^3U) positions [170,175-177]. The SL cap 4, the primary exon sequence and/or pseudouridylation have all been implicated in kinetoplastid *trans* splicing [170,178-181] and polysome association [170,182].

RNA splicing is carried out by the spliceosome, which consists of five small spliceosome nuclear ribonucleoprotein particles (snRNPs) (U1, U2, U4, U5 and U6) as well as non-snRNP proteins. In the human system, there are approximately 45 distinct spliceosomal snRNP proteins and up to 170 proteins were found to be associated with spliceosomal complexes. In trypanosomatids, all five spliceosomal U snRNAs have been identified [169,183] and there are orthologs of all seven Sm proteins [183,184] and of LSm2 to LSm8 [183,185].

Reservosomes

Reservosomes are endocytic organelles of parasites from the Schizotrypanum sub-genus, such as *T. vespertilionis*, *T. dionisii* and *T. cruzi*. In *T. cruzi*, reservosomes are round electron-dense compartments with a mean diameter of 500 nm mainly localized at the posterior region of epimastigote forms. Reservosomes, which were first described as multivesicular bodies [186], present a matrix made primarily of proteins where internal vesicles, membrane profiles, electron-lucent inclusions of a lipid nature and rod-shaped electron-lucent structures bound by a membrane monolayer can be observed [136,187]. Reservosomes are the main site for the storage of proteins and lipids that are ingested by endocytosis and for secretory proteins produced by the parasite [136,188,189]. Reservosomes contain lysosomal proteins (including two peptidase, cruzipain [190-192] and serine carboxypeptidase [193,194]) and an arylsulfatase activity detected by ultrastructural cytochemistry [195]. Due to the presence of peptidases, an acidic pH of 6.0 maintained by the action of two P-type H^+-ATPase isoforms [196] and the presence of Tc Rab11, a homolog of mammalian Rab11 [197], the organelle is also considered the main site of protein degradation and recycling.

Because multiple studies have failed to identify a molecular marker for this organelle, some researchers decided to perform a subcellular proteomic analysis of a purified epimastigote reservosome fraction using several biochemical analysis, including mass spectrometry (LC-MS/MS) [192,198]. Those studies confirmed the presence of the previously described molecules and identified new proteins from different classes such as enzymes, proton pumps and transport proteins. Reservosomes have a complex role in the life cycle of *T. cruzi* directly related to cell differentiation [199]. The unique features of this organelle are potential

targets for the development of chemotherapeutical drugs for Chagas disease.

CHAGAS DISEASE: *TRYPANOSOMA CRUZI*

The pathophysiological aspects of Chagas disease and its mechanism of transmission were first reported by Carlos Chagas in a series of studies published in 1909 [200-202]. After 100 years of research, American trypanosomiasis (or Chagas disease) is still a serious health problem in the Americas. Unfortunately, no vaccines are available and very few anti-parasitic drugs are effective at treating the acute phase of the disease. The most important mode of transmission is through the feces of several species of hematophagous triatomine insects. However, blood transfusion, organ transplantation, congenital transmission and food contamination are other important ways of transmitting the disease [203-205].

The most prominent pathologies associated with the chronic form of the disease are cardiomyopathy and digestive megasyndrome [202,206]. The cerebral form is an infrequent complication of the acute phase that was already mentioned by Carlos Chagas in the original description of the disease [202,206,207]. In addition, it has been shown that chronic Chagasic patients that become immunodeficient because of HIV infection or specific drug treatment may undergo disease reactivation in the central nervous system [206].

Intriguingly, in a series of very elegant experiments, Hecht *et al*. [208] showed the presence of *T. cruzi* DNA in the genomes of Chagas patients and their descendants. Five families with Chagas disease were studied, as confirmed by specific anti-*T. cruzi* antibodies and/or nuclear DNA (nDNA) signatures. The targeted primer-thermal asymmetric interlaced PCR (tpTAIL-PCR) technique was used to demonstrate the rate of lateral DNA transfer (LDT) and to understand the consequences of lateral kDNA transfer (LkDT) from *T. cruzi* to host cells in the families examined. tpTAIL-PCR based on kDNA and long interspersed nuclear element-1 (LINE-1) retrotransposon sequences showed that *T. cruzi* minicircles integrated primarily into host genome transposable elements. Furthermore, the integrated minicircle fragments were inherited by Chagas disease patient progeny. Minicircle integrations into nearly all human chromosomes were detected, kDNA minicircle sequences were concentrated within LINEs and multiple integration events mobilized minicircles and thus moved them to other chromosomes, resulting in disruption of coding regions and gene loss [208]. The LkDT and vertical kDNA transfer (VkDT) events were largely independent, as parasitic kDNA integrations could occur *via* germline or congenital transmission. The authors claim that LkDT- and VkDT-induced genotypic and phenotypic alterations might explain the variability of some clinical manifestations of Chagas disease [208].

Endemic Chagas disease affects eight to ten million people worldwide and kills more than any other parasitic disease in Latin America [209]. Chagas disease extends through North, Central and South America, from Mexico in the north to Argentina and Chile in the south, affecting 21 countries [210] (Fig. **3**). Chagas disease is becoming a global health

Fig. (3). Geographic distribution of endemic Chagas disease. Chagas disease and its vectors are distributed throughout the American continent and some Caribbean islands. The area with infected humans is shown in red (see text, [http://www.dpd.cdc.gov/]).

problem because of the migration of Latin American people to other regions of the world [211]. Thousands of people infected with *T. cruzi* have been reported in the USA, Canada, several European countries, Australia and Japan. In the USA, it was estimated that in 2007, approximately 340,000 Latin American immigrants were potentially infected with *T. cruzi*. Of these, 65,000 may have or develop symptoms or signs of chronic Chagas disease [211].

The genome of *T. cruzi* was recently sequenced and it has been estimated that 50% of the genome is composed of repetitive sequences, consisting mostly of large gene families of surface proteins, retrotransposons and subtelomeric repeats [212]. *T. cruzi* exhibits extensive intraspecific genetic diversity [213] and its population structure has been separated into two major groups, *T. cruzi* I and *T. cruzi* II [214], based on several biological and molecular markers such as isoenzyme analysis, polymorphisms in the 24Sα rDNA and mini-exon gene sequences. *T. cruzi* I strains, which contain zymodeme Z1, are associated with the sylvatic cycle of transmission and arboreal mammals and show low parasitism in human Chagas patients.

In contrast, *T. cruzi* II strains, which contain zymodeme Z2, are associated with the domestic cycle of transmission and a terrestrial niche and cause human infection with high parasitemia in traditionally endemic areas [215]. Studies of the genetic composition and population structure of *T. cruzi* are still quite controversial. The biological properties of the parasite, including its geographical distribution, host specificity and clinical outcomes of infection, have been considered in the classification scheme. Some authors have suggested that *T. cruzi* I and II can occasionally form stable hybrids, sometimes polyploids, that are able to produce regular epidemiological samples in hybrid zone niches [216].

The life cycle of *T. cruzi* consists of two stages in the insect vector and two stages in the human host [202]. In the insect vector, epimastigote forms replicate in the midgut and are then transformed into infective metacyclic trypomastigotes. These forms can be expelled together in the insect's excreta during a blood meal and reach the host's bloodstream through the bite wound or exposed ocular or oral mucosa. In mammals, trypomastigotes are contained within a structure known as the parasitophorous vacuole from which they subsequently escape to differentiate into amastigotes and freely replicate in the cytosol [217]. After several binary divisions, amastigotes differentiate back into highly motile trypomastigotes, which are released upon host cell rupture. During blood feeding, a triatomine insect can acquire the parasite from an infected individual and continue the cycle [36] (Fig. **4**).

Mesenchymal cells, especially macrophages and fibroblasts, are the first cells to be infected by metacyclic trypomastigotes at the site of primary infection [218]. Blood-

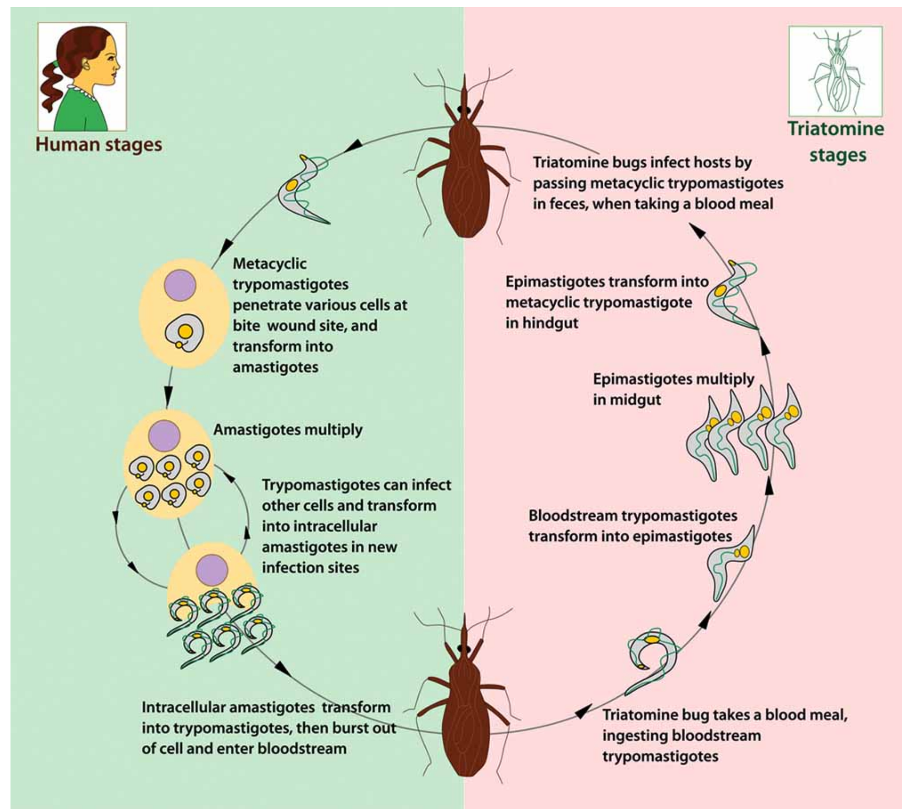

Fig. (4). Life cycle of *Trypanosoma cruzi*. Infection occurs when infected metacyclic trypomastigotes enter the body through wound openings or mucous membranes. The trypomatigotes enter various cells, differentiate into amastigotes and multiply intracellularly. The amastigotes differentiate into trypomastigotes which are then released back into the bloodstream. The life cycle is continued when a reduviid bug feeds on an infected person and ingests trypomastigotes in the blood meal (see text, [http://www.dpd.cdc.gov/]). Diagram based on Stuart *et al.* [217]. (Animated-life cycles of *T. brucei*, *T. cruzi* and *Leishmania* spp. can be seen at the following site: http://www.who.int/tdrold/ media/multimedia/lifecycle.htm).

stream trypomastigotes can then infect several different types of cells farther from the wound site, but stable infection usually occurs in cardiac and skeletal muscle and enteric nerves [219]. Strain-dependent, tissue-specific tropism and genetically distinct strains and clones can be isolated both from animal models and patients with primary cardiac or gastrointestinal disease [220-224].

Prior to entry, parasites must survive, reach the cell surface and form stable attachments to host cells [224]. Interactions of parasites with host cells and the extracellular matrix occur through a diverse group of parasitic surface glycoproteins and peptidases [224]. These surface molecules perform important roles such as binding to host cells, cleaving receptors or ligands, digesting matrix constituents, assisting with immune evasion and triggering bidirectional signaling events in the parasite and host cells [41,204,224,225].

The major cell surface molecules expressed by kinetoplastids, including *T. cruzi*, are glycosylphosphatidylinositol (GPI)-anchored glycoconjugates. Nearly 50% of the *T. cruzi* genome is dedicated to encoding GPI-anchored proteins, which are expressed in all developmental stages and encoded by numerous members of multigene families, including the *trans*-sialidase (TS)/gp85 glycoprotein, mucin, mucin-associated surface protein (MASP) and metallopeptidase gp63 [41,212,226,227]. Enzymatic cleavage *via* GPI-specific phospholipase C (GPI-PLC) releases the head group and has been implicated in lipid and paracrine signaling, as well as signal termination [224,228,229]. Some GPI anchors and GPI-anchored molecules (e.g., TS/gp85 and mucins) in *T. cruzi* are robust proinflammatory molecules that are essential in the modulation of and escape from host immune responses [41,204,226,230-234]. These and several other *T. cruzi* molecules important for binding, entry and survival in host cells are listed in Table **1**.

T. cruzi trypomastigotes directly invade both professional phagocytes and non-phagocytic cells. The cellular mechanisms of phagocytosis have been well-studied [324-327], showing that tissue resident macrophages are critical targets for early *T. cruzi* infection [224,328,329].

T. cruzi trypomastigotes exploit two distinct modes of host cell invasion by which they gain access to the intracellular environment of mammalian cells: a lysosome-dependent pathway and a lysosome-independent pathway [224,330]. In the first case, the parasite induces an increase in intracellular calcium (Ca^{2+}) when it interacts with the host cell, leading to actin polymerization and microtubule reorganization, as well as the fusion of preformed lysosomes with the plasma membrane [36,238,331,332]. The second pathway involves plasma membrane-mediated invagination and phosphoinositide 3 kinase (PI3K) signaling but is independent of actin polymerization [224,330,333,334].

Diverse molecules on the host cell surface interact with *T. cruzi*, including the mannose receptor, L-selectin and Toll-like receptors (TLRs) [335-338]. TLR2, TLR4 and TLR9 are all involved in the recognition of *T. cruzi* during invasion [231,335,339-341]. *T. cruzi* activates TLR2 and TLR4 *via* molecules rich in GPI or GIPL anchors and activates TLR9 *via* the parasite DNA, stimulating cytokine production by macrophages. TLR2 regulates the entry of *T. cruzi* into macrophages through the activation of PI3K [335,339,340].

Rab GTPases are small guanine phosphonucleotide-binding proteins and regulate the entry of *T. cruzi* into cells *via* the endocytic pathway, vesicle trafficking and different stages of phagosome maturation [335,342]. In their active form Rab proteins regulate the binding of other effector proteins and membrane-membrane fusion of vesicles to the developing phagosome [335,343,344]. Rab5 induces the fusion of early endosomes and Rab7 mediates fusion between late endosomes and lysosomes [335,345-347]. Rab5 binds to the membrane and serves as an anchor for the effector protein early endosomal antigen 1 (EEA1), which collaborates with Rab5 during membrane fusion [335,348].

Newly formed parasitophorous vacuoles containing trypomastigotes and amastigotes have an acidic pH. After two hours of infection, 70% of the parasites can be found within partially destroyed vacuoles or free in the cytoplasm. When the pH of the vacuole is elevated by incubation with a variety of drugs, however, the escape of the parasites is significantly inhibited [349-351]. *T. cruzi* trypomastigotes secrete the acid-stable hemolytic protein Tc-Tox within the acidic parasitophorous vacuole. Tc-Tox is then incorporated into the phagosome membrane, forming pores that aid in the destruction of this membrane and the consequent escape of parasites into the cytoplasm [299,352]. Recently, *trans*-sialidase (TS) has also been implicated in escape from lysosomes [224]. In fact, trypomastigotes derived from infected mammalian cells express and release 20 times more TS activity than axenic metacyclic trypomastigotes [353].

The most important integral membrane proteins in lysosomes are lysosome-associated membrane protein 1 and 2 (LAMP1 and LAMP2) [354,355]. Both LAMPs are highly glycosylated and rich in sialic acid and they cover about 80% of the interior surface of the lysosome [355,356]. These two proteins are crucial in *T. cruzi* infection of host cells for both entry and intracellular development, as demonstrated by *T. cruzi* infection in LAMP1 and 2 double-knock-out (LAMP1/$2^{-/-}$) fibroblasts, probably because they are the major source of sialic acid for *T. cruzi* [354,356]. Infection studies with *T. cruzi* metacyclic trypomastigotes have supported this hypothesis, as trypomastigotes overexpressing *trans*-sialidase have shown that increased *trans*-sialidase activity is associated with faster parasite escape from vacuoles [353]. Intriguingly, unlike previous expectations [357], the insertion of TcTox seems to be more efficient in parasitophorous vacuoles lacking sialic acid [354].

Both innate immunity and systemic anti-parasite inflammatory responses are initiated by macrophages through epitope processing and presentation. Interestingly, macrophages play a dual role in *T. cruzi* infection by both harboring [358] and limiting the infection [224,359-361]. *T. cruzi* infection elicits intense innate and adaptive immune responses and focal areas of inflammation. *T. cruzi* can induce the production of cytokines that decrease the expression of molecules critical for T-cell stimulation such as major histocompatibility complex (MHC) class II and costimulatory molecules, possibly as a strategy for survival in the host [362,363]. On the other hand, exacerbated immune responses, although efficient in eliminating the pathogen, may lead to tissue

Table 1. Some Molecules Synthesized by *Trypanosoma cruzi* Involved in Parasite Binding, Entry or Survival in Mammalian Host Cells

Molecules	Activity/Target	Reference
Mucins	Parasite protection and establishment of persistent infection	[234-236]
***Trans*-sialidase family**		
TS and TS-e	*Trans*-sialidase/sialidase	[237-239]
TS1	Binds to β-galactose	[240,241]
Trans-sialidase-like family		
Tc85/Gp85	Binds to laminin and other extracellular matrix components	[226,242,243]
Complement regulatory proteins (CRP)	Bind to mannose; complement regulation	[244-246]
Tc13	Interacts with host beta-adrenergic receptor (?); induces immune responses associated with pathology	[247,248]
Gp82	Mediates metacyclic trypomastigote entry by triggering Ca^{2+} mobilization in both host cell and parasite	[249,250]
Gp90	Binds to mammalian cells in a receptor-mediated manner	[249]
F1-160 (Flagellum-associated protein)	Surface protein that mimics a mammalian nerve protein	[251,252]
Mucin-associated proteins (MASP)	Parasite survival (?)	[212,253,254]
Peptidases		
Cruzipain	Cystein peptidase, bradykinin signaling, generates kinin; favors parasite invasion	[191,255,256]
TcGP63 (*T. cruzi* GP63 related metallopeptidases)	Expressed in all life stages; attachment and/or entry of host cells	[227,257]
Oligopeptidase B	Triggers calcium release, mediates host cell invasion, disassembles host cell F-actin	[258-261]
MMP9 and MMP2 (Matrix metallopeptidases)	Hydrolyze extracellular matrix compounds in host cells	[262,263]
POP/Tc80 (Prolyl oligopeptidase)	Binds to human collagen; fibronectin	[264-266]
Antioxidants		
$T(SH)_2$ (Trypanothione)/ TXN (Tryparedoxin)	Scavenge H_2O_2, peroxynitrite and radiation-induced radicals	[267-273]
Glutathione peroxidase (TcGPXI-cytosolic and glycosomal; and TcGPXII-Endoplasmic reticulum)	Decompose organic peroxides, prevent cellular damage due to lipid peroxidation	[270,271,274]
Trypanothione synthetase	Increases during epimastigote-metacyclic trypomastigote differentiation, virulence factor	[275,276]
Tryparedoxin peroxidases (cTXNPx-cytosolic; mTXNPx-mitochondrial)	Decompose H_2O_2, peroxynitrite and organic hydroperoxides, increase during epimastigote-metacyclic trypomastigote differentiation, parasite survival, replication and differentiation, virulence factor	[275-279]
Ascorbate-dependent hemoperoxidases (TcAPX)	Decompose H_2O_2 but not organic hydroperoxides, potential drug target	[272]
Iron superoxide dismutase (Fe-SOD) mitochondrial, cytosolic and glycosomal	$O_2^{\cdot-}$ detoxification; enzyme overexpression causes increased resistance to complement-dependent programmed cell death and enhanced sensitivity against benznidazol and gentian violet	[280,281]
Heat Shock Proteins		
HSP70	Induces an increase in the secretion of cytokines and growth factors, stimulates programmed cell death in host cells	[282-286]
HSP90 (HSP83)	Controls parasite cell division	[287]
HSP40	Acts with HSP70 and HSP90 as a complex; HSP70 regulators	[288-291]
SHSP16 - Small heat shock proteins (α-HSPs)	Increased by heat stress, biological role (?)	[292]

(Table 1). Contd…..

Molecules	**Activity/Target**	**Reference**
Other molecules		
Mucin-like glycoproteins	Major acceptors of sialic acid on the surface of metacyclic trypomastigotes	[233,293-295]
Proline racemase	B-cell polyclonal activation induced by parasite; favors *T. cruzi* invasion and differentiation	[296-298]
Tc-TOX (Hemolisin)	Forms pores in phagosome, favors parasite escape from phagosome into the cytosol	[299]
Ssp3 and Ssp4 (Parasite surface antigen)	Stage-specific epitopes, favor *T. cruzi* invasion	[300,301]
Amastin	Amastigote surface protein; biological role (?)	[302,303]
Tc PI-PLC (*T. cruzi* phosphoinositide phospholipase C)	Developmentally regulated in amastigotes, differentiation of trypomastigotes into amastigotes	[304-307]
TolT	Bloodstream trypomastigote surface protein; differentiation	[308]
Gp83	Modifies human heart cell receptor, enhancing binding and invasion	[228,309-311]
Penetrin	Binds to heparin/heparan sulfate glycosaminoglycans; promotes trypomastigote attachment to and penetration into host cells	[312,313]
Tc-1 (*T. cruzi* Caseine Kinase II substrate)	Cell surface casein kinase substrate, stage specific, the first described transmembrane surface protein involved in trypomastigote-host cell interaction	[314]
LLGP-67 (Lectin-like 67 kDa glycoprotein)	Protein with galactose-binding activity involved in the recognition of host cell receptors; favors *T. cruzi* invasion	[315]
LYT1 (Lytic pathway protein)	Involved in cell lyses, parasite infectivity and differentiation *in vitro*	[316]
TcMIP (Secreted peptidyl-prolyl *cis-trans* isomerase)	Involved in cell invasion	[317]
Ecto-ATPases	Parasite growth and macrophage infection	[318,319]
Membrane transport proteins	Take up nutrients, establish ion gradients, efflux metabolites, intracellular trafficking, take up or export drugs	[47,320,321]
TXA_2 (Thromboxane A_2 synthesized by *T. cruzi*)	Controls parasite proliferation and modulates the inflammatory responses to the parasite infection	[322]
TcPAF (Platelet-activating factor-like phospholipid synthesized by *T. cruzi*)	Enhances parasite differentiation and mouse-macrophage infection	[323]

pathology [364]. According to Tarleton [365], there are two possible explanations for the delayed generation of protective immune responses: (a) a failure of prompt innate recognition of *T. cruzi* and (b) "immune confusion" generated by the presentation of a wide array of potential target epitopes to $CD8^+$ T cells. The initial innate immune response against *T. cruzi* is mediated in part by TLRs [341]. Among the cytokines generated during this response, interleukin 12 (IL-12), interferon γ (IFN-γ), tumor-necrosis factor α (TNF-α) and type I IFN participate in resistance to the parasite [365]. On the other hand, both TGF-β and IL-10 prevent the protective action of IFN-γ [366]. It was previously demonstrated that membrane components shed by *T. cruzi* increase tissue parasitism and inflammation by stimulation of IL-4 and IL-10 synthesis and this mechanism may play a central role in the pathogenesis of acute-phase Chagas disease [367].

The GPI anchors of mucins from cell-derived trypomastigotes (tGPI-mucins) induce production of the proinflammatory cytokines IL-12 and TNF by interacting with TLR2, TLR1 or TLR6 on the surface of macrophages [234]. Cruzipain is responsible for enhancing IL-4, IL-5 and IL-10 production while decreasing NO production by downregulating iNOS expression and generating endogenous kinins [368]. During infection of its mammalian host, *T. cruzi* secretes a proline racemase (*Tc*PRAC) that contributes to parasite immune evasion by acting as a B-cell mitogen. Overexpression of *Tc*PRAC leads to an increase in parasite differentiation into infective forms and subsequent penetration into host cells [297]. In addition, other molecules released by *T. cruzi* have been shown to cause specific immunosuppression and could be present in vesicles. AgC10, a *T. cruzi* mucin-like protein present in amastigotes, blocks IL-2 synthesis at the transcriptional level by inhibiting tyrosine phosphorylation during T cell receptor-associated signal transduction [369].

Currently, there are no vaccines available for Chagas disease. *T. cruzi* antigens recognized by immune sera from infected humans or animals were the first disease antigens to be described. Characterization of these antigens allowed studies with recombinant proteins based on isolated, antigen-specific genes. Different antigens alone and mixtures of distinct adjuvants, plasmid DNA and, more recently, recombinant viruses and bacteria have been tested as *T. cruzi* vac-

cines [370]. Various antigens delivered using distinct delivery systems have been able to induce protective immune responses in a mouse model of *T. cruzi* infection as measured by a reduction in acute-phase parasitemia, tissue parasitism and mortality [370]. In general, the diversity of the immunodominant response among strains presents a significant challenge for the development of vaccines. A vaccine would require a number of $CD8^+$ epitopes that cover not only the different human MHC haplotypes but also the different parasite strains. Alternatively, the use of sub-dominant epitopes could favor protective immune responses without requiring as many epitopes [370].

The discovery that stem cells are capable of differentiating into specialized cell types has opened new avenues for the treatment of degenerative and traumatic disorders, including heart failure. For Chagas disease, in mice chronically infected with a myotropic Colombian *T. cruzi* strain that received bone marrow cells to repair the heart tissue, the transplanted cells showed a nearly 60% reduction in fibrosis two months after therapy [371]. Although the results of phase II and phase III clinical trials in patients with Chagas disease are encouraging, we are still in the beginning phases of development for a new therapy [372]. Interestingly, a case of chronic Chagas disease and systemic lupus erythematosus was recently described that required immunosuppression to control the autoimmune response. Interestingly, benznidazole induced a reduction, but not elimination, of circulating *T. cruzi* levels and subsequent treatment with posaconazole led to a successful outcome of the infection, even with the use of immunosuppressive therapy [373].

The drugs currently used to treat Chagas disease are nifurtimox, which is derived from nitrofuran and benznidazole, a nitroimidazole derivative. Nifurtimox and benznidazole are trypanocidal to all forms of the parasite [374]. They act through the formation of free radicals and/or electrophilic metabolites but can cause systemic toxicity.

Thus, studies are aimed at finding new therapeutic agents against *T. cruzi* such as inhibitors of certain cellular components, including (a) compounds that interfere with purine metabolism (e.g., allopurinol and purine analogs), (b) inhibitors of ergosterol synthesis, (c) compounds that act in the respiratory chain, (d) inhibitors of alkylphospholipid synthesis (e.g., miltefosine and phospholipid analogs), (e) inhibitors of enzymes involved in nucleotide synthesis, (f) inhibitors of the enzyme trypanothione reductase (e.g., nitrofuran, naphiloquinone and phenothiazine derivatives), (g) inhibitors of cruzipain, (h) inhibitors of glyceraldehyde-3-phosphate dehydrogenase and (i) inhibitors of protein kinases of *T. cruzi* [375]. Recently, K777 (*N*-methyl-piperazine-urea-FhF-vinyl-sulfone-phenyl), a cruzipain inhibitor in preclinical development, has shown good efficacy against different organisms, including *T. cruzi* [376].

Natural compounds with potential antichagasic activity have also been described, including (a) the antioxidative flavanols catechin, epicatechin, gallocatechin, epigallocatechin and some of their gallates; (b) xanthones; (c) tetracyclic triperpenes; and (d) naphthylisoquinoline alkaloids [375]. Further studies are required, however, to better understand these compounds.

Recently, successful elimination of *T. cruzi* transmission by *Triatoma infestans* was reported in Brazil, Uruguay, Chile and parts of Argentina, Bolivia and Paraguay. Still, we should not consider the problem solved because other countries have not implemented a national program for the control of *T. cruzi* transmission and possible re-infestation of treated areas and eventual spread to neighboring regions may take place [377]. Moreover, insecticide control has not been a complete success, especially because strains resistant to pyrethroids have been found in *Rhodnius prolixus* from Venezuela and in *Triatoma infestans* from Brazil [378,379]. Furthermore, in the last decade, different levels of pyrethroid resistance have been detected in an area ranging from northern Argentina to central Bolivia [378-380].

In this scenario, efforts and methodologies need to be combined to reach the prevention of and a cure for Chagas disease, which unfortunately still seems to be a long distance away.

AFRICAN TRYPANOSOMIASIS: *TRYPANOSOMA BRUCEI*

African trypanosomiasis (AT) is caused by protozoan parasites of the genus *Trypanosoma*. It is transmitted by tsetse flies (genus *Glossina*) and is known to be invariably fatal if untreated [381]. African animal trypanosomiasis (AAT) threatens about 50 million head of cattle per year, causing about 3 million deaths, which has a severe impact on cattle production in sub-Saharan Africa [382]. According to the World Health Organization (WHO), 60 million people in 36 countries are at risk of contracting human African trypanosomiasis (HAT, also known as sleeping sickness) and there are about 500,000 people infected with the disease, with at least 70,000 new cases per year [210].

AAT is caused by a large number of species: *Trypanosoma congolense*, *T. vivax*, *T. evansi* and *T. brucei brucei* cause "nagana" in cattle and *T. equiperdum* causes "dura" in horses. Representative strains of *T. equiperdum* and *T. evansi* have been characterized by numerous molecular and classical parasitological approaches and actually shown to be strains of *T. brucei*. Interestingly, these trypanosomes lost part (dyskinetoplastidy - Dk) or all (akinetoplastidy - Ak) of their kDNA [383]) and should therefore be considered two subspecies, *T. brucei equiperdum* and *T. brucei evansi*, respectively, that recently arose spontaneously [383].

Since the discovery of AAT (1894) and HAT (1910), peaks of epidemics have devastated the African continent [384]. Although a noticeable decrease in the incidence of AT was observed between 1949 and 1965, the disease has re-emerged over the past few decades to become one of the major causes of morbidity and mortality in humans and cattle [385]. Thus, AT represents an obstacle to human welfare and cattle rearing, affecting approximately one-third of Africa's total land area (Fig. **5**).

HAT transmission occurs through injection of the infective form of the parasite, metacyclic trypomastigotes (Mts), into the host's skin by the bite of a tsetse fly. A local skin lesion known as a "chancre" may develop 5–15 days later. After this period, Mts transform into bloodstream trypomastigotes and are carried to other sites throughout the body where they reach other blood fluids and continue replication

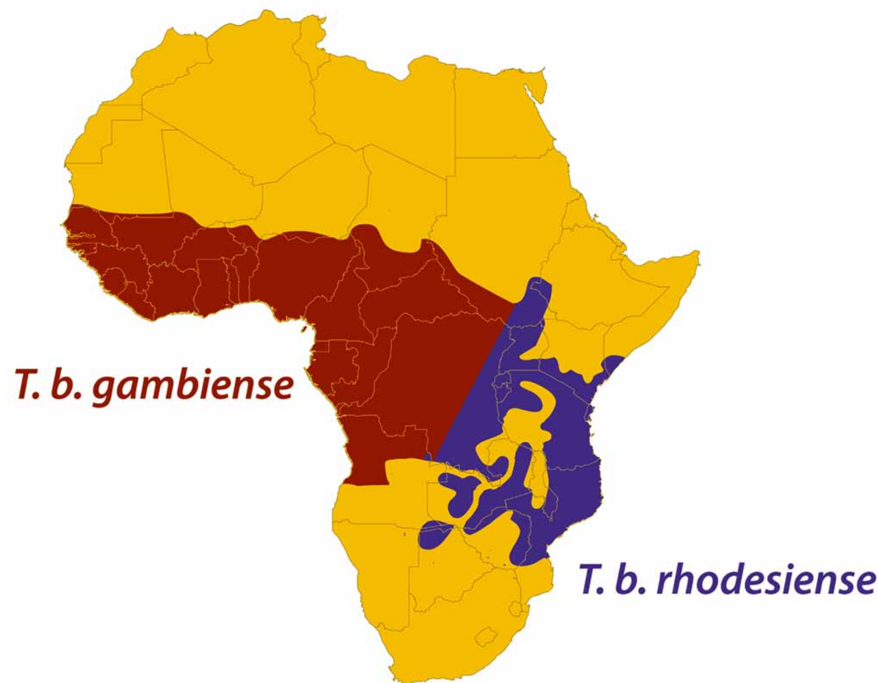

Fig. (5). Geographic distribution of the main subspecies of *Trypanosoma brucei*. Sleeping sickness occurs at a low level of transmission in the majority of countries in tropical Africa, with occasional epidemic outbreaks and at least 50 million people are at risk. Animal trypanosomiasis deters the breeding of domestic stock over large areas of the continent. The area with infected humans and animals is shown in red (see text, [http://www.dpd.cdc.gov/]).

by binary fission. These parasites can cross the blood-brain barrier and invade the central nervous system (CNS) in a few weeks. Tsetse flies are infected with bloodstream trypomastigotes when they suck the blood of an infected mammalian host. In the fly's midgut, parasites transform into procyclic trypomastigotes and multiply by binary fission. They then leave the midgut, transform into epimastigotes, reach the salivary glands, multiply by binary fission and transform into metacyclic trypomastigotes. This complete cycle in the fly takes approximately three weeks [217] (Fig. **6**).

In the early stage of HAT, parasites are found in the bloodstream and lymphatic system (trypanosome proliferation) and symptoms include fever, chills, headache and lymphadenopathy. Immune activation is evident from lymph node enlargement, hepatomegaly and splenomegaly. Later, patients progress to the CNS stage, where the symptoms include severe headaches, insomnia, progressive mental deterioration, psychiatric manifestations and tremors. If left untreated, the disease finally culminates in seizures, somnolence, coma and death [217]. HAT is caused by infection with the morphologically indistinguishable subspecies *T. brucei rhodesiense* (in East and Southern Africa) and *T. b. gambiense* (in West and Central Africa). Over 90% of all reported cases of HAT are caused by *T. brucei gambiense* in which progression to late-stage disease may take several months or longer and late-stage CNS infection may last several years [386,387]. *T. b. rhodesiense* accounts for less than 10% of all reported cases of HAT but is responsible for the most virulent form of the disease with progression to late-stage disease occurring in a matter of weeks and late-stage CNS infection usually leading to death within 3 months [381,388].

As all stages of African trypanosomes occur extracellularly, the parasites have evolved means to evade the host's innate and adaptive immune responses in the plasma mostly through antigenic variation involving variable surface glycoprotein (VSG) [389]. The surface of the parasite is covered with a dense layer of VSG homodimers (circa 10^8 VSG molecules) attached to the trypanosome cell membrane *via* a GPI anchor [390]. *T. brucei* has about 1,000 VSG genes and pseudogenes and of these, only one is transcribed at a time from one of multiple telomeric VSG expression sites [391,392]. The mechanism of antigenic variation in trypanosomes consists of continuous random switching of VSG genes, enabling the parasite to maintain a state of chronic infection in the host that can last for years. The importance of this process is indicated by the fact that VSG genes occupy 10% of the trypanosome genome [393,394].

VSG is involved in several mechanisms of escape from the host immune response such as the prevention of complement activation [389] and reduction of antibody titers against VSG, which occurs by the endocytosis of antibody-VSG complexes, followed by subsequent proteolysis of the antibody and recycling of the VSG back to the parasite surface [74]. VSG is an immunodominant antigen capable of eliciting both T-cell-dependent and -independent B-cell responses, depending on its conformation [395].

Mouse models of infection have shown that both host and parasite genetic factors may control the development of HAT. These factors interact in the immune response to infection, especially in the regulation of macrophage activation and inflammatory responses [396]. The first response of the host immune system consists of classically activated macrophages secreting pro-inflammatory molecules such as TNF,

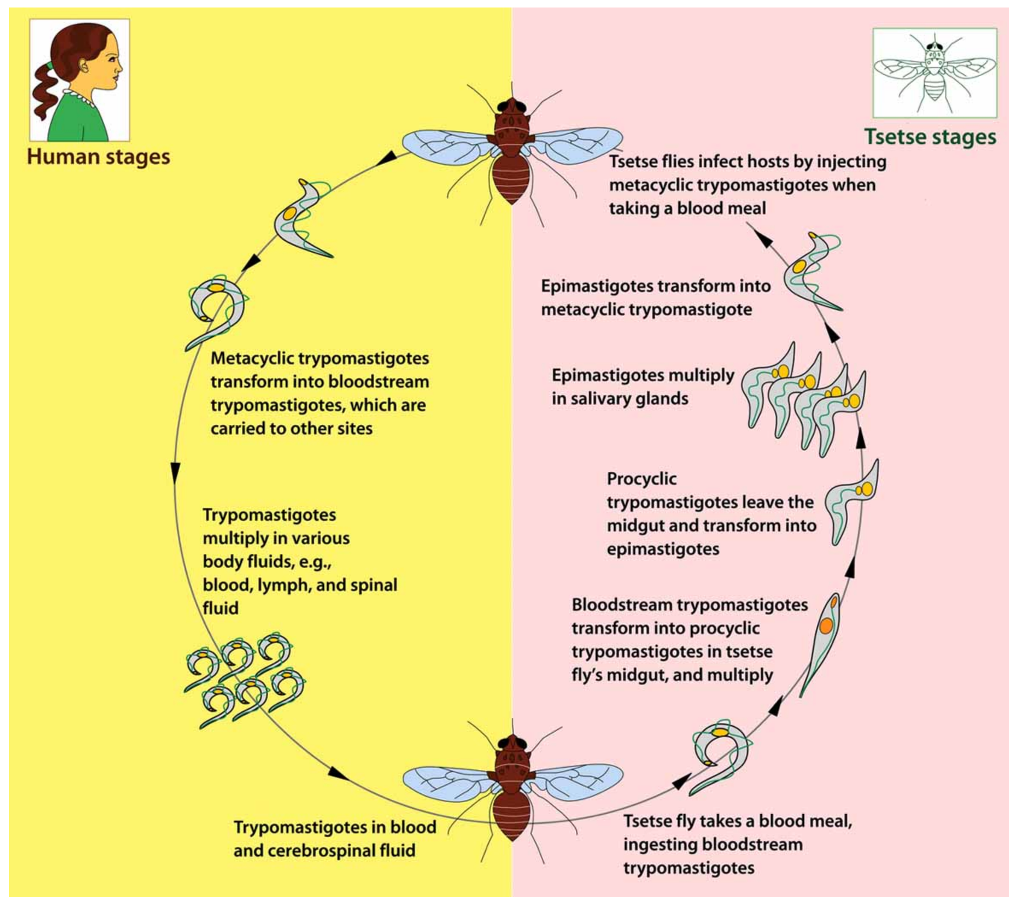

Fig. (6). Life cycle of *Trypanosoma brucei*. During a blood meal on the mammalian host, an infected tsetse fly (genus *Glossina*) injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream. Inside the host, they transform into bloodstream trypomastigotes, are carried to other sites throughout the body, reach other blood fluids (e.g., lymph, spinal fluid) and continue the replication by binary fission. The entire life cycle of African Trypanosomes is represented by extracellular stages. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission. The cycle in the fly takes approximately 3 weeks. Humans are the main reservoir for *Trypanosoma brucei gambiense*, but this species can also be found in animals. Wild animals are the main reservoir of *T. b. rhodesiense* (see text, [http://www.dpd.cdc.gov/]). Diagram based on Stuart *et al.* [217]. (Animated-life cycles *T. brucei*, *T. cruzi* and *Leishmania* spp. can be seen at the following site: http://www.who.int/tdrold/media/multimedia/lifecycle.htm).

IL-1, IL-6 and NO [397]. The GPI anchor of VSG also interacts with macrophages (*via* a presumed receptor) and induces the secretion of pro-inflammatory cytokines [390,398,399]. Interestingly, throughout both human and animal trypanosomiasis, TNF plays an important role in the control of parasitemia, as well as in the pathology resulting from infection (e.g., anemia, neurological disorders, fever and cachexia) [397,400]. VSG has been identified as the primary molecule inducing TNF in trypanosome-soluble extracts [397]. Apparently there are two modes of action for VSG: the glycosylinositolphosphate (GIP) fraction directly induces macrophage activation and the induction of TNF-α in macrophages following IFN-γ stimulation, whereas the dimyristoylglycerol (DMG) component of the anchor is not able to induce TNF-α directly but is involved in macrophage priming [397].

A long, drawn-out inflammatory response can cause pathology; therefore, it is vital for the host to reduce inflammation by downregulating the classic activated macrophages and their pro-inflammatory cytokines. Longer survival of the host relies on the production of type II cytokines such as IL-4, IL-10 and IL-13, which can induce macrophages to become more anti-inflammatory [401-404]. Comprehensive immune suppression affects both humoral (B cell) and cellular (T-cell and macrophage) immune functions [405], leading to trypanosome-induced immunopathology [406-408]. A frequent pathological trait is anemia, the degree of which is a sign of disease severity [409].

The main feature of HAT is a striking increase in immunoglobulin (Ig) levels, particularly IgM, including both trypanosome-specific antibodies and non-specific Ig production induced by cytokine activation of B cells [410]. In African trypanosomiasis, the VSG-specific B cell responses can occur in a T-cell independent manner, although T-cells improve the B-cell response primarily by secreting cytokines that mediate antibody class switching [411]. From this perspective, increased IL-4 mRNA levels and a concomitant increase in IgG1 antibodies against VSG have been observed

in trypanotolerant cattle infected with *T. congolense*, but not in trypanosusceptible cattle [404]. In animal trypanosomiasis, trypanotolerance involves the combination of a humoral response, which is necessary to control parasitemia, with the ability to oppress immunopathology [397]. Additionally, B-cells become suppressed or exhausted later in the infection, resulting in a total absence of IgG responses and a greatly reduced IgM response [397,412].

The humoral response to VSG has immunopathological consequences, e.g., the generation of auto-antibodies induced by molecular mimicry [413] and immune complex disease [414]. Furthermore, in the late CNS stage of infection, both trypanosome-specific IgG and IgM and polyclonal IgM responses have been detected in cerebrospinal fluid, which may be due to modified plasma cells in the white matter or plasma cells that form perivascular infiltrates in the brain [387,414].

In addition to antigenic variation, African trypanosomes cause a loss of various B cell populations, disable the host capacity to raise a long-lasting and specific protective anti-parasite antibody response and abrogate vaccine-induced protective responses to non-related human pathogens [415].

Even more than 100 years since the discovery of African trypanosomes, only a few clinically useful drugs have been developed. These drugs are still considered unsatisfactory, however, mainly due to significant toxicity and severe side effects [217].

In the early stage of HAT, two drugs, pentamidine and suramin, are used against *T. b. gambiense* and *T. b. rhodesiense*, respectively. Pentamidine, an aromatic diamidine, has been used since the 1930s and acts by inducing changes in DNA topology and inhibiting topoisomerases, resulting in impaired DNA replication. Some adverse effects have been reported, however, such as nephrotoxicity and diabetes mellitus [416]. Suramin, a polysulfonated naphthalene derivative, was first used against sleeping sickness in 1922. The trypanocidal action of suramin is possibly due to inhibition of low density lipoprotein (LDL) uptake, affecting the parasite's supply of cholesterol and phospholipids. Unfortunately, this drug causes several side effects, mainly involving allergic reactions and renal complications [417].

For late-stage disease, the drugs of choice are melarsoprol (active against both *T. b. gambiense* and *T. b. rhodesiense*) and eflornithine (active only against *T. b. gambiense*). Melarsoprol, an arsenic derivative, has been used since 1949 and is the main drug of choice to date [418], although its mechanism of action remains unknown [419]. On the other hand, severe side effects have been observed with melarsoprol such as convulsions and other neurological sequelae that can precede coma and death in the reactive encephalopathy that afflicts 5-10% of treated patients [420] and is fatal in 10-70% of cases [421].

In contrast, eflornithine, an analog of the amino acid ornithine that acts by inhibiting the polyamine biosynthetic enzyme ornithine decarboxylase (ODC), is relatively safe. This drug has a similar affinity for both mammalian and trypanosomal ODCs, but it is degraded much more slowly in trypanosomes [418]. Recent clinical trials have shown that combinatorial therapy with eflornithine and nifurtimox is quite promising against the late/cerebral stage of *T. b. gambiense* infection [418,421].

The sequenced genome of *T. brucei* emphasized that generating a vaccine for AT is highly unlikely due to the alternate expression and recombination of a repertoire of approximately 1,000 genes encoding VSG [217,422]. On the other hand, characterization of invariable surface glycoproteins (ISGs) is an appealing alternative [394,423]. For instance, one of the ISG antigens has been used in DNA vaccine preparations against an experimental model of AT and shown to elicit humoral responses, achieve partial immune protection and preferentially induce Th1-like IgG2a anti-*T. brucei* antibodies. Furthermore, a DNA vaccine encoding a *trans*-sialidase gene induced partial immunoprotection against *T. b. brucei* in mice [424]. Preliminary data on vaccination with GPI in mice indicate that TNF-associated immunopathology is reduced in subsequently infected mice [390]. In other words, an understanding of the immunological components of this disease may offer new opportunities not only for vaccine development but also for therapeutic intervention [387].

LEISHMANIASIS: *LEISHMANIA* SPP.

Leishmaniasis is one of the major insect-borne diseases in developing countries. *Leishmania* species are found in most inter-tropical and temperate regions of the world. Transmission occurs through the bite of the insect vector, the phlebotomine sandfly. Leishmaniasis currently threatens 350 million people in 88 countries globally. Worldwide, 2 million new cases are estimated to occur annually and 12 million people are presently infected [210] (Fig. **7**).

Leishmaniasis refers to a group of diseases that can be divided into cutaneous, diffuse cutaneous, mucocutaneous and visceral leishmaniasis; visceral leishmaniasis (VL) is lethal if untreated. Cutaneous leishmaniasis (CL) affects the skin, causing ulcers that usually heal after some weeks; the mucocutaneous form (MCL) causes ulceration, followed by the destruction of mucous membranes of the nasal, oral and throat cavities and surrounding tissues. The diffuse cutaneous form (DCL) produces disseminated and chronic skin lesions and it is more difficult to treat. Cases of DCL in Bolivia, Brazil and Peru account for 90% of all of the cases worldwide. The most dangerous form of leishmaniasis, however, is VL. It is characterized by high fever, weight loss, anemia and swelling of the liver and spleen [425]. Currently, another important aspect of the disease is co-infection with the human immunodeficiency virus (HIV); in these co-infection cases, the risk of development of VL increases by 100 to 1000 times [426].

The genus *Leishmania* was created in 1903 by Ross. Species of this genus are identified primarily by their clinical manifestation and geographic distribution. Other important criteria for classification include behavior of the parasite in sand fly and mammalian hosts [427], as well as biochemical and molecular characteristics [24,428-430]. More than twenty species have been described and allocated into two subgenera: *Leishmania* and *Viannia* [427,431]. The former contains species from both the Old World and the New World and the latter is composed only of species from the New World [432].

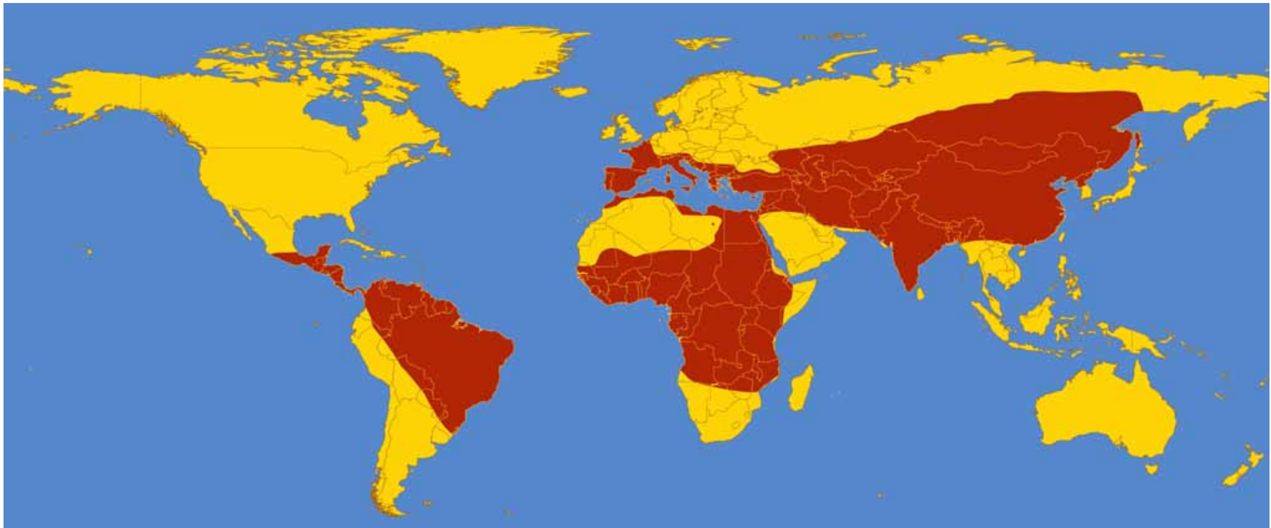

Fig. (7). Geographic distribution of endemic leishmaniasis. Leishmaniasis is found in parts of about 88 countries. Approximately 350 million people live in these areas. Most of the affected countries are in the tropics and subtropics. The settings in which leishmaniasis is found range from rain forests in Central and South America to deserts in West Asia. More than 90 percent of the world's cases of visceral leishmaniasis are in India, Bangladesh, Nepal, Sudan and Brazil. Leishmaniasis is found in Mexico, Central America and South America, from northern Argentina to Texas (not in Uruguay, Chile, or Canada), southern Europe (leishmaniasis is not common in travelers to southern Europe), Asia (not Southeast Asia), the Middle East and Africa (particularly East and North Africa, with some cases elsewhere). The areas with infected humans are shown in red (see text, [http://www.dpd.cdc.gov/]).

The life cycle of *Leishmania* involves three developmental forms: the amastigote, the procyclic promastigote (Fig. **1A**) and the metacyclic promastigote [433]. The sandfly vector becomes infected when feeding on the blood of an infected individual or an animal reservoir host. In the sandfly, *Leishmania* parasites replicate as extracellular, actively motile, elongated (10-20 micrometers), flagellate procyclic promastigotes, which primarily inhabit the insect's alimentary tract. Procyclic promastigotes are multiplicative forms, not infective to mammalian hosts and found in the midgut. Metacyclic promastigotes are non-dividing forms, shorter than the procyclic promastigotes (7 to 10 micrometers), infective to mammalian hosts and found in the thoracic midgut and proboscis of the sandfly. When an infected sandfly feeds on a mammalian host, its proboscis pierces the skin and saliva-containing anti-coagulant is injected into the wound to prevent the blood from clotting; the metacyclic promastigotes are transferred to the host along with the saliva. Once in the host, the metacyclic promastigotes differentiate (after being phagocytosed by a macrophage) into the intracellular amastigote form (Fig. **1C**). This form of the parasite, round and non-motile (3 to 7 micrometers in diameter), resides within the parasitophorous vacuole (a vacuole with lysosomal features), where it resists the microbiocidal action of the acid hydrolases from the lysozymes; the amastigote form survives and multiplies inside these vacuoles, eventually leading to lysis of the macrophages. The released amastigotes are taken up by additional macrophages, so the cycle continues. Ultimately, all the organs containing macrophages and phagocytes are infected, especially the spleen, liver and bone marrow [433] (Fig. **8**).

The major components of the surface coat are free GPI glycolipids and/or bound to the cell surface *via* GPI. These components form protective layers that mediate host-parasite interactions; the constitution of this surface coat is precisely regulated during the course of the parasite life cycle [40]. Lipophosphoglycans are the most abundant macromolecules on the surface of *Leishmania* promastigotes and contain a structurally distinct GPI anchor [434]. Free GPIs constitute the major class of parasite molecules on the surface of amastigotes. However, amastigotes also acquire glycosphingolipids from the macrophage host; these glycosphingolipids appear to be incorporated into the exoplasm of the amastigote plasma membrane [40]. In fact, *Leishmania* species present many surface macromolecules, including GIPL and lipophosphoglycan (LPG), as well as the membrane proteins proteophosphoglycan (PPG), MSP/GP63 (major surface protein or 63-kDa glycoprotein), PSA-2/GP46 (promastigote surface antigen-2 or 46-kDa glycoprotein) and amastin surface proteins (developmentally regulated amastigote proteins that are unique to the Trypanosomatidae). These glycoconjugates perform an important role in parasite adaptation and establishment in different hostile environments, such as the sandfly midgut and the macrophage phagolysosome [257,435,436].

Neutrophils are short-lived leukocytes that are first recruited to inflamed tissues and thus play a crucial role in immunity to infection [437]. Neutrophils die by apoptosis, necrosis and NETosis [438], when they release fibrous traps of DNA, histones and granule proteins named neutrophil extracellular traps (NETs). These NETs attract and kill *Leishmania* efficiently, as previously described for fungi and bacteria [437].

To survive, *Leishmania* must evade activation of the mammalian host's immune responses. In the macrophages, the parasites undergo many rounds of replication and produce a great number of amastigotes [63]. During infection, upon activation, macrophages produce pro-inflammatory cytokines, such as interleukin 12 (IL-12), which induces the activation of Th-1 responses to combat intracellular parasites

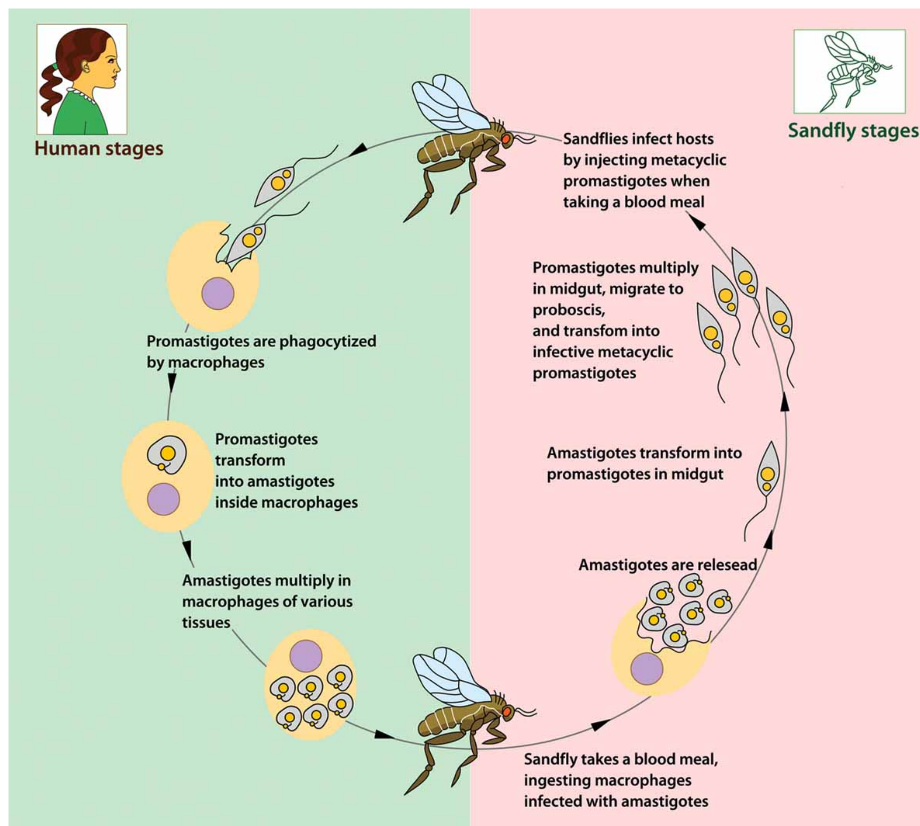

Fig. (8). Life-cycle of *Leishmania* spp. Leishmaniasis is transmitted by the bite of infected female phlebotomine sandflies. The sandflies inject the infective promastigotes from their proboscis during blood meals. Promastigotes that reach the wound are phagocytized by macrophages and other types of mononuclear phagocytic cells. Progmastigotes transform into amastigotes, which multiply by simple division and proceed to infect other mononuclear phagocytic cells. Parasite, host and other factors affect whether the infection becomes symptomatic and whether cutaneous or visceral leishmaniasis results. Sandflies become infected by ingesting infected cells during blood meals. In sandflies, amastigotes transform into promastigotes, develop in the gut and migrate to the proboscis (see text, [http://www.dpd.cdc.gov/]). Diagram based on Stuart *et al.* [217]. (Animated-life cycles *T. brucei*, *T. cruzi* and *Leishmania* spp. can be seen at the following site: http://www.who.int/tdrold/media/multimedia/lifecycle.htm).

[63]. Th-1 lymphocytes induce the activation of macrophages by secretion of INF-γ, which stimulates macrophages to produce nitric oxide, which is the major player in killing intracellular parasites [439]. The importance of TLR4 in the recognition of *L. major* promastigotes has been proposed [440]. Increased number of parasites in *L. major*-infected TLR4$^{-/-}$ mice was shown to be associated with an overall increase in Th1- and Th2-like cytokine production *in vitro* [440]. On the other hand, a global decrease in both Th1- and Th2-like cytokines (IFN-γ and IL-10) was observed in *L. pifanoi*-infected WT and TLR4$^{-/-}$ mice [441].

There is still a need for innovative and alternative therapies against leishmaniasis. Despite decades of investigation, an efficacious vaccine against human leishmaniasis has not yet been developed, as attempts to confer significant protection against human leishmaniasis have ultimately failed [217,426]. On the other hand, the efficacy of a heterologous prime-boost vaccination using attenuated vaccinia virus and *Leishmania* DNA expressing an antigen homolog of receptors for activated C kinase has been shown [442]. Additionally, the first vaccine against canine leishmaniasis, Leishmune® vaccine, was recently licensed in Brazil. This vaccine presents around 76 to 80% effectiveness and has also been shown to be valuable in blocking transmission, causing a 79% reduction in the capacity for transmission by sandflies previously fed on serum of vaccinated dogs. In addition, this vaccine was also shown to effectively treat dogs infected with the parasite; at 22 months post-vaccination, 90% of the animals were asymptomatic [443-445].

The drugs currently used for the treatment of both VL and CL are the pentavalent antimonials, sodium stibogluconate and meglumine antimoniate. These drugs require long-term parenteral administration and are very toxic and almost obsolete. The antibiotic amphotericin B has been the drug of choice for the treatment of VL in India due to the emergence of drug-resistant parasites [217]. Miltefosine, a phospholipid derivative and the first oral treatment for VL, is effective against CL and was registered for treatment of both VL and CL in India and Colombia in 2002 and 2005, respectively. However, its use is limited because of potential teratogenicity [217,446]. Several groups of synthetic phospholipids have also shown activity against *Leishmania* and other protozoa [217,447].

Molecular targets need to be validated as crucial for parasite growth or survival using gene knockout or knockdown technologies and/or using highly specific small molecule inhibitors. Current estimates suggest that about 10% of

known genes are able to bind drug-like small molecules [217].

PLANT INFECTIONS: *PHYTOMONAS* SPP.

Phytophagous insects have generally been regarded as the primary vectors of *Phytomonas* spp. [448,449]. When a competent insect vector feeds on infected plant fluid, *Phytomonas* spp. reach the intestinal tract and, after migrating throughout the hemocele, reach the salivary glands. As the infected insect feeds on another plant, the flagellates are then transmitted *via* saliva [449-450]. Plant flagellates might also circulate between different insects, passing from one insect to another probably by coprophagy or during group feeding, common behaviors among insects [449,451] (Fig. **9**).

After natural transmission in fruits and seeds, plant flagellates remain concentrated near the point of inoculation [448]. In lactiferous plants, the infection is generally limited and not all ducts are infected [452]. In contrast, flagellates that infect phloem vessels may disseminate throughout the vascular bundle [453]. Whereas the infection of fruits, seeds and latex tubes is not harmful, the infection of phloem sap is generally associated with lethal disease [453,454]. The pathology promoted by *P. francai* in *Manihot palmata* (*esculenta*), popularly known as cassava or manioc, results in chlorosis of the leaves and considerable atrophy of the roots, causing producers to incur major losses [449].

Little is known about the geographical distribution of species of the genus *Phytomonas*. Thus far, it has been recognized that the genus *Phytomonas* is endemic in South America, with most species isolated from Brazil; in a few European countries, particularly in Spain and France; in Asia, particularly in India and China; and in Northwest Africa [455,456] (Fig. **10**). These findings suggest that the genus *Phytomonas* may be dispersed globally and strains from several other places in the world may be isolated within the next few years.

Vickerman and Preston [459] proposed that only trypanosomatids with a digenetic life cycle in plants and insects that retain the promastigote form throughout should be considered *Phytomonas*. On the other hand, a century after the first description of the genus, clear criteria to define plant isolates at the species level are still not available. A feature that is very common among *Phytomonas* promastigotes, but not commonly observed in insect trypanosomatids, is a corkscrew shape of the cellular body [460-462]. Another peculiar feature present in all *Phytomonas* species studied to date is extreme variation in shape and size depending on whether they grow in a host (plant or insect) or in axenic medium [463]. These changes in the form and shape of *Phytomonas* promastigotes may be the result of physiological changes that these trypanosomatids undergo in their hosts. All currently known plant trypanosomatids have been grouped in the genus *Phytomonas*, although they can differ greatly in terms of both their biological properties and their effects on the host [463].

When analyzed by electron microscopy, plant flagellates show the standard ultrastructure displayed by the majority of trypanosomatids. Perhaps the only unique trait of the cellular organization of plant flagellates is the pronounced quantity of glycosomes [106]. In all trypanosomatids, a large part of

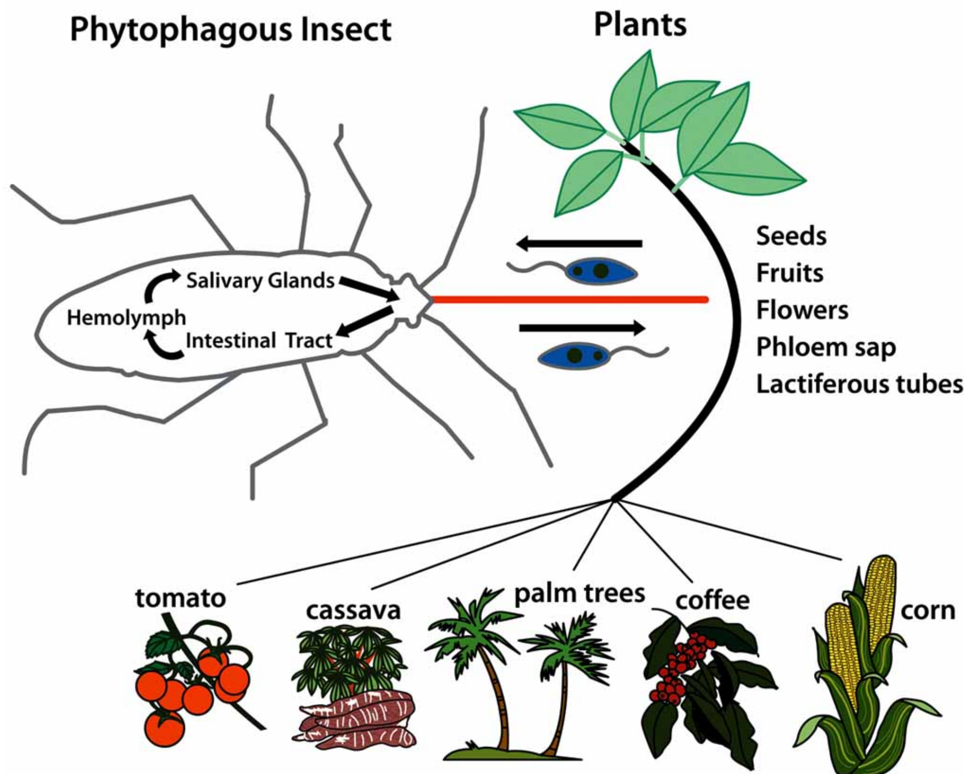

Fig. (9). Life cycle and transmission of *Phytomonas* spp. Parasites ingested by phytophagous insects reach the intestinal tract and pass through the hemocele, migrating through the hemolymph to infect the salivary glands. The infected insect transmits the parasites *via* saliva when it feeds on another plant. The major plant cultures affected by *Phytomonas* spp. are shown.

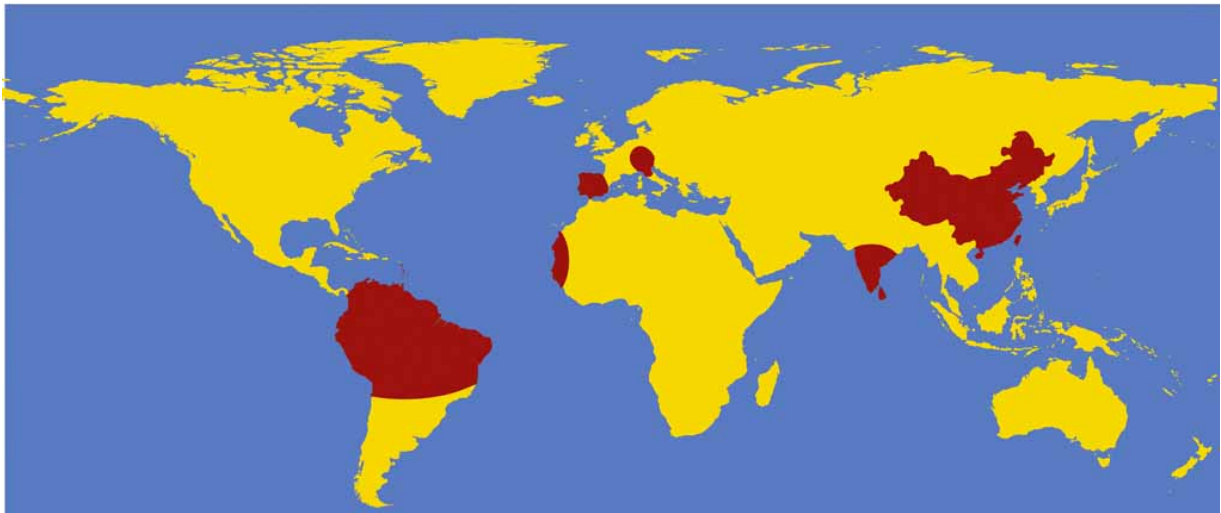

Fig. (10). Geographic distribution of *Phytomonas* spp. The map shows the distribution of *Phytomonas* spp-infected plants (in red) (see text [http://www.dpd.cdc.gov/]). Diagram based on Sturm *et al.* [456], Dollet *et al.* [457] and Votypka *et al.* [458].

glycolysis is performed in glycosomes [119]. The importance of these organelles for *Phytomonas* spp. is extremely pronounced once the plant stage is reached given that the mitochondria do not contain a functional Krebs cycle, cytochromes or the classical respiratory chain and the parasites are not able to catabolize amino acids; therefore, ATP production is based on glycolysis [464-466]. Respiration in these parasites is insensitive to inhibitors of ubiquinol-cytochrome *c* oxidoreductase (respiratory Complex III or *bc*1) and cytochrome *c* oxidase (respiratory Complex IV) [467]; however, respiration is entirely sensitive to salicylhydroxamic acid (SHAM), an inhibitor of cytochrome-independent trypanosome alternative oxidase (TAO) [138]. TAO performs a terminal oxidation step in a multicomponent pathway that serves to re-oxidize NADH produced during glycolysis [467]. This metabolic pattern is similar to that observed in a long, slender bloodstream form of *Trypanosoma brucei*, whereas in *Phytomonas* spp., it is observed in culture [467].

The plant stage of *Phytomonas* spp. involves the secretion of enzymes that the utilization of cellulose, starch, sucrose and xylulose, carbohydrates present in large quantities in the phloem, latex and fruits [12]. *P. serpens* possesses peptidases that share biochemical and biological functions with the metallopeptidase gp63 and cysteine peptidases, which are virulence factors present in *Leishmania* spp. and *T. cruzi*, respectively [257,468-471]. The importance of these peptidases in the interaction of *Phytomonas* species with the salivary glands of insect vectors has been demonstrated [468,472].

Recent studies have demonstrated immunological similarities between *P. serpens* and *T. cruzi*. For instance, serum samples from Chagas disease patients showed strong reactivity against *P. serpens*. In addition, the immunoprotective proprieties of *P. serpens* antigens were clearly demonstrated in mice previously immunized either orally or intraperitoneally with *P. serpens* that then showed reduced parasitemia and an increase in survival after being challenged with a known lethal inoculum of *T. cruzi* [473].

The genus *Phytomonas* has been considered a monophyletic group more closely related to insect trypanosomatids and *Leishmania* than to the genus *Trypanosoma* [2,24,474-476]. On the other hand, recent wide evolutionary divergence among members of the genus *Phytomonas* has been described and extremely different genomic organization between two *Phytomonas* groups has been observed [477,478]. Most notably, the chromosome number was found to be 7 in one *Phytomonas* group (with a genome size of 10 Mb) versus 21 in another group (25 Mb). These data demonstrate unsuspected genomic diversity among plant trypanosomatids that may justify further debate about their division into different genera [478].

PERSPECTIVES

The governments and pharmaceutical companies still regard parasitic diseases as neglected diseases. Considerable progress made by control programmes in some endemic localities contrasts with persisting difficulties in other vast geographic regions. Recent emergence of the diseases in non-endemic areas because of population migrations has become a problem. Drugs for treatment of these diseases are limited, poorly tolerated and not very effective, especially with raising numbers of drug resistance. On the other hand, the scientific community has grown to appreciate the importance of parasites as models for studying evolution, speciation and other questions of broader significance in biology. Molecular epidemiology and phylogeography, combined with insightful laboratory experiments, have transformed our understanding of the diseases caused by *T. cruzi*, *T. brucei* and *Leishmania*. Intriguing questions remain to be answered and new techniques, especially for developing better drugs, need to be improved.

ACKNOWLEDGEMENTS

We dedicate this review to Maria de Nazareth Silveira Leal de Meirelles, who has always been an outstanding adviser. We wish to express our gratitude to Mr. João Müller Haddad, who kindly made the diagrams depicted in this review. The studies performed in our laboratories were supported by the Brazilian financial agencies Conselho Nacional

de Desenvolvimento Cientifico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa Carlos Chagas Filho (FAPERJ) and Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular.

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Received: November 15, 2009 Revised: September 21, 2010 Accepted: September 22, 2010

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