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MOLECULAR AND CELLULAR BIOLOGY OF PATHOGENIC TRYPANOSOMATIDS

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Editors:
Marcelo Santos da Silva
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Frontiers in Parasitology

(Volume 1)

Molecular and Cellular Biology of Pathogenic Trypanosomatids

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FOREWORD

Since 1970, due to important financing programs, Brazilian scientists have been able to practice Biology while studying trypanosomatids, mainly *Trypanosoma cruzi* and *Leishmania* spp. The Annual Meeting in Caxambu since 1974, focusing on *Trypanosoma cruzi* at the beginning, and later extending the interest to other parasites, particularly *Leishmania,* were the catalysts of a scientific boom in Brazilian biology. Many young and enthusiastic students have been trained by the pioneers in the morphology, physiology, biochemistry, immunology and molecular biology of trypanosomatids, including vectorial transmission and clinical aspects of the diseases. The Caxambu meetings also brought to Brazil renowned scientists of the field who established important and long-lasting connections with Brazilians. After 2005, the field has gained momentum after the publication in Science of the complete genome of three species of the order Kinetoplastida, namely *Leishmania major*, *Trypanosoma cruzi* and *Trypanosoma brucei*. This e-book is a consequence of the Brazilian scientific activities in the field in the last 45 years. Maria Isabel Cano is my scientific grand-daughter and Marcelo Santos da Silva got his Ph.D. under her supervision.

The first chapter deals with the cellular organization of trypanosomatids during the life cycle. This group diverged early in evolution and due to this fact their members conserved certain characteristics not found in other eukaryotes as organelles like glycosomes, reservosomes and acidocalcisomes, among other cell compartments. Differences between species and between the distinct life cycle forms of each organism are comprehensively discussed.

Chapter 2 unveils the fascinating area of the trypanosomatid genomes. Information has been made available by the Tritryps project that provided major insights into the genome structure and organization of these parasites. While *T. brucei* presents subtelomeric expansion of genes related to antigenic variation, *T. cruzi* and *Leishmania* genomes contain species-specific genes related to cellular invasion and survival inside the mammalian host cells. As control of gene expression operates mainly at a post-transcriptional level in trypanosomatids, gene copy number variation is probably an efficient mechanism to enhance gene expression and increase sequence variability. Among the Tritryps, *T. cruzi* presents the most striking expansion of species-specific multigene families, which could be related to the ability of the parasite to infect any nucleated cell of a broad range of mammals. Chromosomal copy number variation is also well tolerated by these parasites, allowing the expansion of a whole set of genes simultaneously. The functional implications of these chromosomal expansions to the parasite biology are still to be determined.

Chapter 3 clarifies the function of chromosome ends and telomeres in trypanosomatid biology. These structures, in addition to the preservation of chromosomal integrity, play a

major role in survival. The telomeric repeat $(5'\text{-TTAGGG-}3')_n$ is conserved among trypanosomatid species, but adjacent subtelomeric sequences vary between species and chromosomes within the same cell. For example, size and gene content of *T. cruzi* subtelomeres differ in each chromosome due to differences in the abundance and organization of these genes, whereas in *Leishmania* spp. subtelomeres show a more conserved organization.

Chapter 4 is a pleasant travel on nuclear and kinetoplast DNA replication in trypanosomatids. These organisms exhibit both conserved and unique non-conserved features in the DNA replication machinery. Curiously, the trypanosomatid pre-replication complex differs from other eukaryotes, having features similar to those of Archaea. The completion of DNA replication, at trypanosomatid telomeres, apparently is similar to other eukaryotes, although the processing of the leading and lagging telomeres required to generate the 3' overhangs, which serve as telomerase substrate, remains unknown. DNA replication in trypanosomatids initiates almost simultaneously in the nucleus and the kinetoplast, suggesting that regulation of DNA synthesis in the two DNA-containing organelles may be coordinated. The kinetoplast DNA consists of mini- and maxi-circles, which are replicated by many proteins with, as yet, unknown mechanisms of action. The complex DNA replication mechanisms, independently acting in both kinetoplast and nucleus, are reviewed.

Chapter 5 discusses mechanisms by which trypanosomatids maintain genome integrity and preserve faithful DNA replication despite multiple environmental aggressions. A growing body of evidence on how trypanosomatids recognize and repair damages is reviewed.

Chapter 6 explores one of the most fascinating features that make trypanosomatids unique organisms in the biological world. Despite being eukaryotic organisms they constitutively synthesize polycistronic mRNAs from separate gene clusters. Control of gene expression is not carried out at the DNA level but relies upon post-transcriptional mechanisms. This chapter aptly discusses the common post-transcriptional pathways for most genes, although many regulatory strategies within species of the group differ from each other. It is argued that these complex and diversified regulatory machineries allow rapid responses of these organisms to drastic environmental changes during their life cycle.

Chapters 7 and 8 describe virulence factors and the immune evasion in *Leishmania* spp and *Trypanosoma cruzi*, respectively. These parasites co-evolved with their hosts – mammalian and insects – for several millions of years and developed specialized strategies to evade the immune system by overcoming both innate and adaptive immune responses. The role of different species of molecules as virulence factors is discussed.

Diagnosis, chemotherapy, and potential recombinant vaccines are discussed in the ensuing

chapters 9, 10 and 11, respectively. The diseases caused by kinetoplastids, called neglected diseases since they are predominant in poorer tropical countries having scarce resources, are responsible for thousands of deaths per year. No vaccines are available for these diseases. Presently, drug therapies are not very effective because the few available drugs are toxic, and treatment is costly. Comprehensively, methods of vector control are insufficient, despite the Southern Cone initiative in the nineties by which transmission of *Trypanosoma cruzi* in the domicile and outdoors by *Triatoma infestans,* at least in some countries, has been controlled. Chapter 9, stresses the need for reliable and specific diagnostic tests for epidemiological surveys, surveillance for vectorial transmission, blood screening, screening of pregnant women and their newborns, and in individual patients. The chapter summarizes the most commonly used molecular tools described to date to detect *T. cruzi* infection and to identify and genotype *Leishmania* spp. Chapter 10 discusses the present status of the chemotherapy area, emphasizing the screening assays that led to a few drugs reaching the stage of a clinical trial after a selection from millions of molecules tested. A milestone has been set for the year 2020, by the London Declaration: control of Chagas disease and leishmaniasis, and elimination of Human African Trypanosomiasis. Advances to achieve these goals are presented in this chapter. Finally, Chapter 11 deals with the research efforts on the development of recombinant vaccines against trypanosomatids, most of which are at the stage of preclinical experimentation. It is hoped that these efforts can be translated into efficient human vaccines.

This book is being edited in electronic form. In addition to the PDF edition accessible online, it will be aired in open access electronic media. Any reader can also have access to a printed version and also to individual chapters offered by the authors themselves. Thus, it is hoped that biologists, graduate students and post-doctoral researchers benefit with this up-to-date and competent review of the present literature on trypanosomatids.

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PREFACE

Among the pathogenic trypanosomatids are the etiological agents of leishmaniasis, African trypanosomiasis and Chaga's disease, protozoa parasites belonging respectively, to the genus *Leishmania* and *Trypanosoma*. The diseases are high prevalent in tropical areas of the globe and according to WHO cause thousands of new cases and deaths every year. It is worth noting though that international migration made Chaga's disease and leishmaniasis becoming an issue also in many developing countries in Europe, Canada, USA, Australia, and Japan. Although the number of new cases and deaths from Chaga's disease had decreased in the last decade, the millions of chronically infected persons who are at risk for developing cardiovascular and/or digestive pathology make Chaga's disease one of the leading causes of cardiovascular morbidity and premature death in Latin America. Human African trypanosomiasis (HAT), also known as sleeping sickness, is endemic in sub-Saharan countries and is caused by *T. brucei gambiense* and *T. b. rhodesiense*. The number of HAT cases has been decreasing in the last years due to very active control efforts although according to a recent WHO report, country or regional averages may be misleading since the burden of the disease falls very heavily on some areas. HAT patients require lots of care and disease diagnosis and treatment can be costly and time-consuming. However, in many Africa countries diagnostics and HAT drugs are provided free of charge. Leishmaniasis in its turn is still considered a dangerous menace, principally to the poor, with about 12 million people currently infected. Depending on the species leishmaniasis can be expressed in different clinical forms, with the cutaneous form being the most common, causing self-limiting skin ulcer or a highly disfiguring scar, to the disfiguring and mutilating mucocutaneous form, and the visceral form is the most severe and fatal if not treated. All these diseases are still challenges to overcome since the absence of effective vaccines, and the toxicity of current anti-parasite drugs, in addition to the emergence of drug-resistant parasite strains and HIV co-infections are non-transposable barriers to disease control. Thus, many research initiatives have been direct to understand parasite biology and its interactions with different hosts, as well as the mechanisms of disease pathogenesis, of drug resistance and genome organization and maintenance, with the aim of the development of more efficient diagnostic tools and non-toxic and effective drugs and vaccines.

In this eBook, experts review and explore current knowledge about the molecular and cellular biology of trypanosomatids, highlighting the most important and actual discoveries in each research field. Topics covered include cell organization during development; genome organization and maintenance; control of gene expression; nuclear and kinetoplast DNA replication; mechanisms of DNA damage repair; virulence factors and immune evasion; new methods for molecular diagnosis; new therapeutic tools and recombinant vaccine biology.

We believe that the eBook content will be of keen interest to undergraduates, graduate students and principally to the Parasitology community and researchers working in related fields.

We would like to thank the contributing authors of this book for their time, expertise, and for making this eBook novel, educational, and informative and Professor Walter Colli for written the Forward.

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CHAPTER 1

The Cellular Organization of Trypanosomatids During Life Cycle

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Abstract: *Trypanosoma cruzi* , *Trypanosoma brucei* and *Leishmania* spp. are etiological agents of the following neglected diseases: African sleeping sickness (*T. brucei*), Chagas' disease (*T. cruzi*) and leishmaniasis (*Leishmania* spp.). These parasites are eukaryotic cells that diverged early in evolution and therefore harbor modified organelles, such as glycosomes, and present subcellular compartments with unusual characteristics. This chapter aims to overview the most striking features of the structures and functions of these organelles, which ensure the existence of these parasites, and to discuss the differences between species and between the distinct life cycle forms of each organism.

Keywords: Acidocalcisomes, Cellular compartmentalization, Endoplasmic reti-

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culum, Flagellum, Glycosomes, Golgi, Kinetoplast, Nucleolus, Nucleus, Reservosomes.

INTRODUCTION

Trypanosomatids are unicellular flagellated eukaryotes that belong to the Kinetoplastida class, the members of which are characterized by the presence of a kinetoplast, which is a structure that contains the mitochondrial genetic material. The Kinetoplastida class includes the Trypanosomatidae family, which comprises human pathogens, such as *Trypanosoma cruzi* , *Trypanosoma brucei* and *Leishmania* spp. These are the etiological agents that cause Chagas' disease, African sleeping sickness and leishmaniasis, respectively. It is currently estimated that 6.5 million people are infected with *T. cruzi,* that there are 1.3 million new cases of leishmaniasis and approximately 6,000 cases of sleeping sickness every year (www.who.int).

These protozoans have life cycles stages that possess different morphologies and cellular structures when living in mammalian and insect hosts. The *T. cruzi* epimastigote is a form that is defined by the lateral exit of the flagellum from the cell body; it is also the non-infective life cycle stage of the parasite. It proliferates *via* binary fission in the gut of *Triatoma infestans* insects, which are more commonly known as kissing bugs. In the insect hindgut, epimastigotes transform into the metacyclic-trypomastigotes in which the flagellum is inserted along almost the entire length of the protozoan. When the insect vector ingests blood from a mammalian host, the trypomastigotes are then eliminated with the feces. Released parasites can penetrate the mammalian host through contact with different mucosal tissues. They enter cells by forming a parasitophorous vacuole, which is then disrupted and the parasites transform into amastigotes, which are spherically shaped and have a very short flagellum. Amastigotes proliferate inside the cell cytosol and then transform into non-replicative trypomastigotes, which are released *via* cell lysis to reach the bloodstream. The life cycle is completed by the ingestion of the blood by insect vectors. The trypomastigotes are able to transform into epimastigotes that replicate inside the insect gut [1].

Leishmania, in contrast, alternates between a promastigote and an amastigote form. Promastigotes are protozoan with a flagellum attached to one extremity that

develops in the digestive tract of sand flies. Promastigotes differentiate into metacyclic forms that are eliminated *via* regurgitation when the insect feeds. The parasites are then phagocytized by macrophages before transforming into amastigotes that divide inside vacuoles. After several rounds of division, the macrophages are disrupted, and the new amastigotes are released to infect adjacent macrophages. Insects ingest the infected macrophages when they feed on mammalian blood. Amastigotes transform into promastigotes in the insect midgut, continuing the life cycle [2].

Unlike what occurs in *T. cruzi* and *Leishmania*, the life cycle of *T. brucei* is entirely extracellular. An infected tsetse fly (*Glossina* spp.) bites a mammalian host; inoculating metacyclic-trypomastigote forms into the circulatory system. The injected metacyclic-trypomastigotes transform into bloodstream trypomastigotes, which then proliferate in the hemolymphatic system as slender trypomastigotes. *T. brucei* species survive the immune defenses of the host by continuously changing their coat, which is formed by a single variant surface glycoprotein (VSG) through a process known as antigenic variation. When a high density of parasites is achieved in the blood, some of these parasites transform in a non-proliferative, stumpy form. This form is able to differentiate into procyclic trypomastigotes when ingested by new tsetse flies. In the fly midgut, the procyclic form proliferates and then migrates to colonize the salivary glands of the insect, where they transform into epimastigotes that can proliferate by binary fission. After some rounds of duplication, the epimastigotes become metacyclic trypomastigotes, which are then injected into a new mammalian host during a tsetse fly's bite [1].

These organisms contain organelles that are common to all eukaryotes, but they also harbor unique organelles, such as the kinetoplast, reservosomes, glycosomes and flagellum-related structures, all of which present peculiar features as a consequence of their earlier evolutionary origins and the requirements of adaption (Fig. **1**).

This chapter aims to present recent developments that have increased our understanding of how these organelles ensure the survival of the organism in different hosts during parasite life cycles. We have also compared the features that

CHAPTER 2

Trypanosomatid Genome Organization and Ploidy

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Abstract: In 2005, the draft genome sequences of the parasites *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*, also known as the Tritryps, were published providing major insights into their genome structure and organization. Even though these parasites diverged around 200 to 500 million years ago, their core genomes are highly syntenic and conserved. These conserved regions are interspersed by retroelements, structural RNAs and species-specific genes related to host-parasite interactions. While *T. brucei* presents a subtelomeric expansion of genes related to antigenic variation, *T. cruzi* and *Leishmania* genomes contain species-specific genes related to cellular invasion and survival inside the mammalian host cells. Duplication events have also shaped the genome architecture of these parasites. As control of gene expression operates mainly at a post-transcriptional level in trypanosomatids, gene copy number variation is probably an efficient mechanism to enhance gene expression and increase sequence variability. These parasites also explore gene conversion mechanisms to generate variants and increase their surface complexity. Among the Tritryps, *T. cruzi* presents the most striking expansion of species-specific multigene families, which could be related to the parasite's ability to infect any nucleated cell of a broad range of mammals. Chromosomal copy number variation is also well tolerated by these parasites, allowing the expansion of the whole set of genes simultaneously. The functional implications of these chromosomal expansions to the parasite biology are still to be determined.

Keywords: Aneuploidy, Chromosomal variation, Chromosomes, Comparative genomics, Copy-number-variation, Genome organization, Genome structure, Multigene families, Polycistron, Synteny.

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INTRODUCTION

Trypanosomatids are a group of kinetoplastid parasitic protozoa that belong to the Trypanosomatidae family, which includes several species that cause disease in humans.

Trypanosoma and *Leishmania* genera stand out due to their importance in public health being the etiological agents of African trypanosomiasis (*Trypanosoma brucei*), Chagas disease (*Trypanosoma cruzi*) and Leishmaniasis (various species of *Leishmania*). These parasites have a digenetic life cycle alternating between invertebrate and vertebrate hosts and present different developmental stages adapted to survive in each of their hosts. Another important characteristic of these organisms is the presence of a network of circular mitochondrial DNA, also known as kinetoplast that is unique in terms of structure, replication and functionality and gives the order Kinetoplastida its name. In the following sections of this chapter, we will provide an overview of the epidemiology, lifecycle, taxonomy and genomic organization of *Leishmania* and *Trypanosoma*.

EPIDEMIOLOGICAL CONTEXT OF THE TRYPANOSOMATIDS

Leishmania: The Causative Agent of Leishmaniasis

Leishmaniasis is a group of complex tropical diseases caused by protozoan parasites of the genus *Leishmania*. These parasites are transmitted by the bite of infected phlebotomine sand flies from the *Lutzomyia* genus *in* the New World (Central and South-America) and *Phlebotomus* in the Old World (East-Africa, the Mediterranean region, and the Indian subcontinent).

Epidemiological studies have shown that leishmaniasis is currently spread in 98 countries on five continents, with 12 million people infected, 350 million people at risk and more than 1.5 million new cases documented each year. The incidence of this disease is increasing due to its progressive adaptation to urban environments, an increase in its dispersion due to human migration, climate change and co-infection with other diseases, especially in poor regions and developing countries (Fig. **1**) [1, 2].

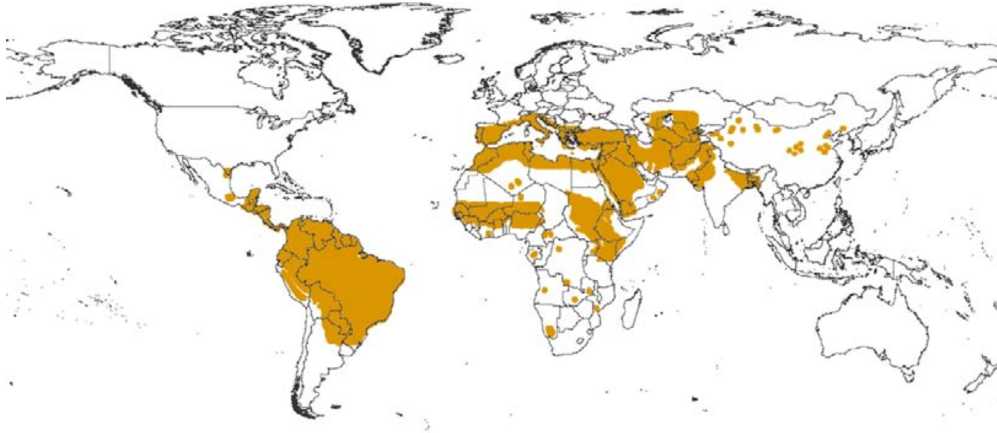

Fig. (1). Geographic distribution of leishmaniasis. Source: World Health Organization, October 2010.

There are at least 20 *Leishmania* species that can cause disease in humans, and are divided into two subgenera: *Leishmania* and *Viannia*. These species are responsible for a wide spectrum of clinical manifestations that are grouped into tegumentary leishmaniasis (TL), and visceral leishmaniasis (VL) (Table **1**) [3].

Table 1. Clinical manifestations of leishmaniasis and associated species.

Clinical Manifestation	Characteristics	Species
Cutaneous leishmaniasis	Papules, nodular lesions with keratotic plates that can ulcerate	*L. (Viannia) braziliensis* *L. (V.) peruviana* *L. (Leishmania) major* *L. (L.) mexicana*
Mucocutaneous leishmaniasis	Lesions in mucosal tissues at the nares, mouth and throat	*L. (V.) braziliensis* *L. (V.) guyanensis* *L. aethiopica*
Diffuse cutaneous leishmaniasis	Disseminated non-ulcerative skin lesions in the body	*L. (L.) amazonensis* *L. (L.) mexicana*
Visceral leishmaniasis	Fever, anemia, weight loss and swelling of visceral organs	*L. (L.) infantum* *L. (L.) donovani*
Post-kala-azar dermal leishmaniasis	Macular or nodular rash after treatment of visceral leishmaniasis	*L. (L.) infantum* *L. (L.) donovani*

TL encompasses three distinct types of diseases that are cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML) and diffuse cutaneous leishmaniasis (DCL),

CHAPTER 3

Chromosomes Ends and Telomere Biology of Trypanosomatids

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Abstract: In this chapter, we focus on the structure and function of telomeres and subtelomeres of human protozoan parasites *T. cruzi*, *T. rangeli* and *Leishmania* spp.. Beyond their role in maintaining the integrity of chromosomes, telomeres and subtelomeres are involved in the survival mechanisms of these single-celled parasites. The telomeric repeat $(5'\text{-TTAGGG-}3')_n$ is conserved among trypanosomatid species, but adjacent subtelomers vary between species and chromosomes within the same cell. The chromosome ends of *T. rangeli*, for example, exhibit a simple organization with short subtelomeres whereas *T. cruzi* subtelomeres are a complex mosaic of genomic fragments including gene/pseudogenes corresponding to large gene families of surface proteins and retrotransposons. Differences in the copy number and organization of these genes determine the variation in the size of subtelomeres on each *T. cruzi* chromosome. *Leishmania* subtelomeres, in contrast, lack genes encoding surface antigens; instead they carry conserved repeat sequences referred to as telomere-associated sequences. *T. cruzi* and *T. rangeli* chromosomes share a high level of synteny which is lost in the subtelomeric regions. It has been suggested that *T. cruzi* subtelomeres can serve as recombination hotspots and thus promoting the increase of the repertoire of surface antigens. Many pieces of evidence indicate that telomere maintenance in Kinetoplastids occurs primarily by a telomerase-mediated elongation.

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The catalytic subunit of telomerase (TERT) is present in all sequenced trypanosomatid species, whereas the RNA component containing a template for telomere repeat extension has recently been identified in *T. brucei* and *Leishmania*. Further studies are needed to understanding the regulation of telomere homeostasis and the biology of subtelomeres of trypanosomatids.

Keywords: Base modification, Chromosomes ends, Contingency genes, Retrotransposons, Subtelomere, Surface protein genes, Telomerase, Telomere generation, Telomere junction, Telomere repeats.

INTRODUCTION

Telomeres are nucleoprotein complexes whose function is to stabilize the physical ends of a linear eukaryotic chromosome, protecting them from nucleases, end-to-end fusions and cellular recombination machinery, ensuring their stability and integrity. Telomeres may also be involved in establishing the spatial architecture of the interphase nucleus. This chapter is addressed to emphasize some recent findings to establish a comparison between telomere of three trypanosomatids species (*Trypanosoma cruzi*, *Trypanosoma rangeli* and *Leishmania*), taking into account the established knowledge from other eukaryotes. It does not contain a compilation of all available data.

OVERALL STRUCTURE OF TELOMERES

A general picture of the structure of trypanosomatid telomeres and subtelomeres is shown in Fig. (**1**). Telomeres consist of repetitive DNA associated with a variety of proteins. Tandem arrays of double-stranded 5’-TTAGGG-3’ hexamers of variable size are found in *T. cruzi* (0.3-0.5 kb) and *T. brucei* (10-20 kb) telomeres [1 - 4]. Toward the chromosomal terminus, the G-rich strand is single-stranded ending in a common 5'-GGGTTAGGG-3' sequence in all *Trypanosoma* species. Moreover, overhangs of G-rich strand in *T. brucei* originate structures known as telomere loops (T-loops) because they may fold back and invade the double-stranded telomeric DNA [5] (Fig. **2**). Telomeres in *Leishmania* are constituted by tandem arrays of the same 5′-TTAGGG-3′ hexamer [4, 6, 7], although in *L. braziliensis* a different telomere repeat of 14 bp 5’-CCCTA-

-CCCGTGGA-3’ is also found in some chromosome ends in addition to the conventional hexameric repeats [6, 8, 9]. Moreover, the telomere overhangs (size range: ≥ 9 nt in *L. major*, *L. donovani* and *Trypanosoma* species; ≤ 12 nt in *L. amazonensis*) differ among some *Leishmania* species and also between *Leishmania* and *Trypanosoma*. The sequence of *L. donovani* and *L. major* 3′G-overhang is 5’-GGTTAGGGT-3’ [4, 7] and GTTAGGGTTAGG-3’ in *L. amazonensis* [10] (Figs. **1** and **2**).

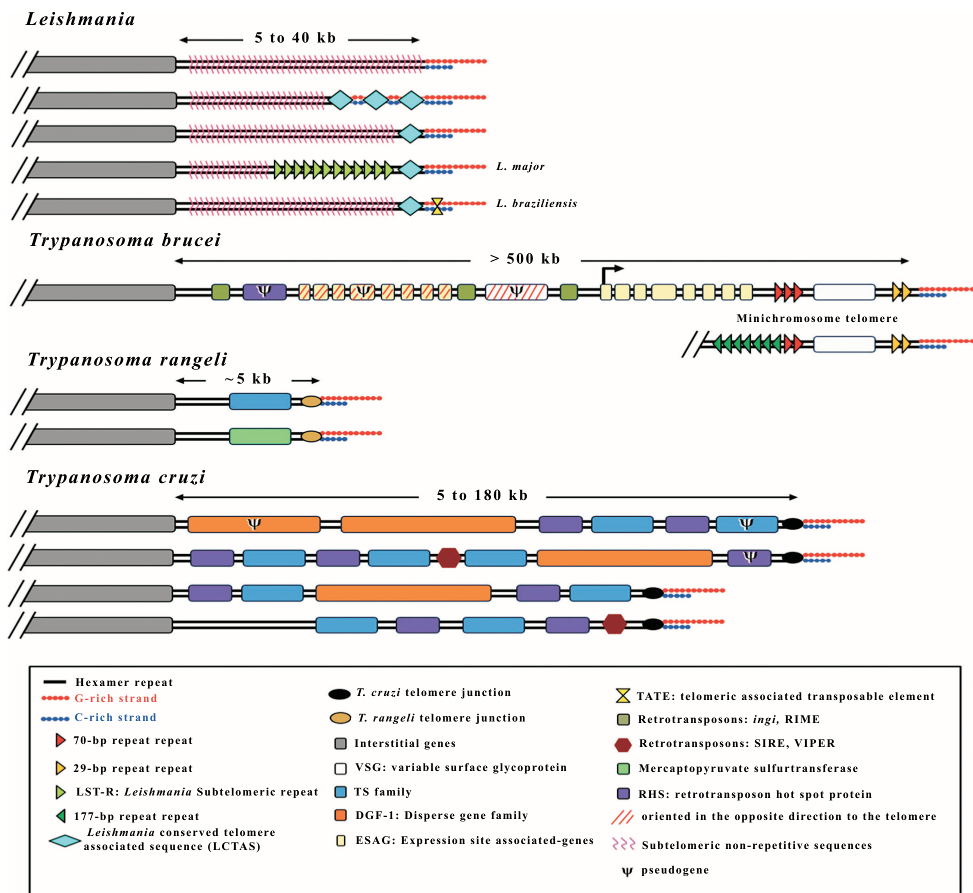

Fig. (1). Schematic representation of trypanosomatid telomeres. Two types of telomeres identified in *T. rangeli* are shown. The heterogeneity of *T. cruzi* subtelomeres is represented with the four most common organizations observed in chromosome ends of this parasite. The size of the subtelomeric region is indicated above each map. Boxes indicate genes and other sequence elements. The maps are not to scale. *T. brucei*, *T. cruzi* and *T. rangeli* maps were adapted from El-Sayed *et al.* [14], Barros *et al.* [15], Stoco *et al.* [11], respectively.

CHAPTER 4

Nuclear and Kinetoplast DNA Replication in Trypanosomatids

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Abstract: Trypanosomatids are among the most primitive eukaryotes and therefore exhibit both conserved and unique non-conserved features in the DNA replication machinery. In eukaryotes, nuclear DNA replication is preceded by the assembly of the pre-replication complex (pre-RC), which is coordinated by the six-subunit origin recognition complex (ORC), which together with the Cdc6 and Cdt1 proteins play a central role in the loading of the hetero-hexamer Mcm_{2-7}. In the domain Archaea there are no Cdt1 protein homologs, Mcm is a homo-hexamer, which is recruited by a protein that shows homology with ORC, and Cdc6 (called Orc/Cdc6). Curiously, trypanosomatid pre-RC differs from others eukaryotes in this context, and it appears more similar to that of Archaea, presenting a homolog of protein Orc/Cdc6 and no homologs of Cdt1, in addition to present Mcm as a hetero-hexamer complex. The completion of DNA replication, at trypanosomatid telomeres, apparently is similar to other eukaryotes, although the processing of the leading and lagging telomeres required to generate the 3' overhangs, which serves as telomerase substrate, remains unknown. With the generation of overhangs at the ends of the chromosomes, telomeres are frequently extended by the action of telomerase, whose control also remains unknown. It is worth mentioning that DNA replication in trypanosomatids initiates almost simultaneously in the nucleus and the kinetoplast, suggesting that regulation of DNA synthesis in the two DNA-containing organelles may be coordinated. The kinetoplast DNA (kDNA) consists of mini- and maxicircles, which are replicated by many proteins

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whose mechanisms of action remain unclear. This chapter aims to review and discuss the complex DNA replication mechanisms that act independently in the kinetoplast and the nucleus, as well as some fascinating peculiarities exclusive to trypanosomatids protozoa group.

Keywords: DNA polymerases, Kinetoplast DNA replication, Nuclear DNA replication, Origin Firing, Origin Licensing, Origin recognition complex, Replication forks, Replisome, Telomere replication, Topoisomerases.

INTRODUCTION

For most eukaryotes the cell cycle can be considered, in general, as two distinct events: DNA replication, or synthesis (the S phase), and mitosis (the M phase), separated by two gap phases (G1 and G2). In trypanosomatids, the cell cycle is a fundamental process, essential for efficient proliferation, even in inhospitable environments, ensuring a correct duplication and segregation of single-copy specialized organelles, such as flagellum, nucleus, and mitochondrion. The duplication of these single-copy organelles requires precise temporal and spatial segregation of certain specialized structures, which trigger a very clear morphological pattern that changes according to the cell cycle. Chapter 1 contains descriptions of trypanosomatids life stages and their morphological changes within their specific hosts. Also, detailed morphological descriptions of *T. cruzi*, *T. brucei* and *L. amazonensis*, show that these morphological patterns are found in each phase of the cell cycle and vary among trypanosomatid species [1 - 3]. In addition, in these parasites DNA replication initiates almost simultaneously in the nucleus and the kinetoplast, suggesting that DNA synthesis is coordinately regulated [3 - 5].

DNA replication is an important biological process due to its importance in the maintenance and storage of genetic information, which is responsible (directly or indirectly) for cellular metabolic control. Moreover, as mentioned in the previous chapter, trypanosomatids alternate between replicative and non-replicative forms during their developmental life cycle, suggesting that the control of replication may be directly involved in the infective ability of these organisms. As these protozoa diverged early in the eukaryotic lineage, unique aspects of their DNA

synthesis can explain how DNA replication mechanisms have evolved.

In this chapter, we will discuss various aspects of DNA replication in the two organelles that contain DNA: nucleus and mitochondrion (kinetoplast). Also, we will explain some of the main functions of DNA polymerases in these protozoans as these are essential enzymes adapted to perform a wide variety of DNA transactions [6, 7].

DNA POLYMERASES

In 1955, Arthur Kornberg began elucidating the fascinating DNA synthesis process. Over two years, Kornberg and his students measured DNA synthesis in *E. coli* extracts, using ^{14}C-labeled thymidine, a known constituent of DNA [8]. Afterward he isolated the first DNA polymerizing enzyme and named it DNA polymerase. In 1959, Kornberg shared the Nobel Prize in Physiology or Medicine with Severo Ochoa, who had demonstrated the mechanisms in the biological synthesis of ribonucleic acid (RNA) [8]. Kornenberg studies paved the way for subsequent studies seeking to elucidate DNA replication mechanisms in Bacteria, Archaea and Eukarya.

DNA polymerases can replicate DNA in both strands, by adding nucleotides continuously on the leading strand and discontinuously on the lagging strand. They play an essential role in DNA replication [9, 10] and are involved in several processes from DNA chain elongation, to recombination, replication, and repair processes [11, 12]. DNA polymerases are divided into seven families according to their sequence similarities (Table **1**). Polymerases γ, θ and Pol I are members of A family. The B family is represented by α, ε, δ and Pol II and Pol III is the sole member of C family. Some members of D family remains uncovered, but here are include some enzymes found in the Archaea. Tdt, β, λ, μ and σ members represent the X family. Y family includes Pol I, η, κ, Pol IV and Pol V. RT family is the last group and includes the reverse transcriptases (RT) in retroviruses, and the eukaryotic RNA-dependent DNA polymerases; both can do the reverse synthesis of RNA into DNA (Table **1**). In most eukaryotes, the RT family is usually restricted to telomerase [13].

CHAPTER 5

Genome Maintenance in Trypanosomatids

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Abstract: The genome is the source of life, providing the information needed to direct all aspects of organismal function. Propagation of life requires copying of the genome and faithful transmission from parent to offspring. Many challenges confront genome propagation, including ensuring the accurate and complete copying of the DNA, circumventing impediments to DNA replication, and maintaining genome integrity in the face of myriad insults and during periods of cellular quiescence. Just as importantly, the genome must be allowed to change, either incrementally through small mutations in sequence or by large-scale rearrangements. Such changes not only drive evolution, but can be integral components of an organism's life cycle. In this chapter we consider the rapidly growing body of knowledge on how the genomes of kinetoplastid parasites are maintained, by describing the range of genome repair and damage tolerance pathways that operate. We focus on *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania*, three important human and animal pathogens, but we believe the lessons learned from the study of genome maintenance in these genetically tractable parasites are applicable widely, not only to other parasites but throughout biology.

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Keywords: Base excision repair, DNA repair, DRP lyase activities, Genome integrity, Homologous recombination, Microhomology-mediated end-joining, Mismatch repair, Non-homologous end-joining, Nucleotide excision repair, Translesion DNA synthesis.

INTRODUCTION

Propagation of life requires transmission of the genome from parent to offspring. A high level of fidelity in transmission allows the retention of selected genetic characteristics. For this reason, complex machineries for highly accurate genome duplication have evolved and are identifiably conserved throughout extant life. However, in addition to replication, accurate genome transmission requires processes that identify and respond to threats to the integrity and content of the genetic information. All genomes are subject to constant attacks, which can arise exogenously, such as from environmental radiation or chemicals, or endogenously, such as due to free radicals generated during metabolism. In cellular organisms, such attacks can lead to DNA damage in both the nuclear and organellar genomes, potentially resulting in genetic alterations, which may be beneficial but are frequently detrimental and may cause death. The forms of damage to DNA genomes are wide-ranging, including modified or lost bases, base mismatches, incorrect intra- or inter-strand chemical bonds and single- or double-strand breaks (SSBs and DSBs). The primary response to damage is mediated by DNA repair processes, which survey the genome for all these forms of damage and, having identified them, correct the structure. Normally, DNA repair ensures that the genetic content is maintained, but occasionally genome sequence or organisation change is an unavoidable by- product of the reactions. Sometimes the repair is not possible and, in these circumstances, damage bypass may be invoked, potentially resulting in an elevated rate of genome change. Indeed, in some circumstances organisms deliberately invoke genome alterations for continued survival, including by harnessing the normally protective functions of the repair processes.

In this chapter we discuss our growing understanding of the diverse array of genome maintenance processes that are active in kinetoplastid parasites, reflecting the wide range of genome damage that the organisms face. In addition, we discuss growing evidence for the absence of at least one key repair process, as well as

kinetoplastid adaptations in what are normally considered highly conserved reactions. Finally, we highlight areas of limited understanding, which may become the focus of future research. The interested reader is referred to recent reviews that provide further insight into these topics [1 - 3].

The Genomes of Kinetoplastids

Kinetoplastids represent an evolutionary grouping of eukaryotic microbes marked by shared, diverged features of core eukaryotic biology. One such divergence, for which the grouping is named, is the mitochondrial genome (the kinetoplast), which is composed of an interlinked lattice of ~1,000 minicircles and ~20 maxicircles [4, 5]. Divergence is not limited to the kinetoplast, however, since nuclear genome sequencing has revealed a highly unusual genetic landscape in which virtually all genes are organised in a relatively small number (~150-200) of so-called directional gene clusters (DGCs), some of which can encompass hundreds of genes [6, 7]. This arrangement reflects diverged RNA Polymerase (Pol) II transcription, in that virtually every gene is expressed from a multigene transcription unit (which, again, can encompass hundreds of genes). Thus, transcription of each gene within a DGC initiates from a single, shared transcription start site and ends at a shared termination site, with coupled trans-splicing and polyadenylation used to generate mature mRNAs from a multigene primary transcript. Initiation and termination sites have been termed strand-switch regions (SSRs), represent only a fraction (~1%) of the number of nuclear genes and are marked by the accumulation of variant histones, modified histones and a modified base (named J) [8 - 16]. It is increasingly clear that the diverged organisation of transcription in kinetoplastids is reflected in gene expression novelties, most notably highly diverged RNA Pol II promoters and the devolution of gene expression controls from transcription initiation to post-transcriptional processes [17, 18]. However, the impact of multigenic transcription is unlikely to be limited to gene expression controls, with wider (and, as yet, less well explored) consequences for genome stability.

Central to the potential impact of multigenic transcription on genome stability is replication of the nuclear genome, a reaction whose machinery and dynamics have only recently begun to be examined [19]. The interaction between transcription

CHAPTER 6

Mechanisms Controlling Gene Expression in Trypanosomatids

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Abstract: As members of a highly divergent group of eukaryotes, trypanosomatids present peculiar mechanisms of gene expression. These protozoan parasites have transcription and processing machineries that constitutively transcribe clusters of non-related genes into polycistronic pre-mRNAs, which are subsequently *trans*-spliced into monocistronic transcripts. Because of this, control of gene expression relies mainly on post-transcriptional mechanisms that are, for the most part, mediated by RNA binding proteins that control steady-state levels of mRNAs and/or their translation rates. Using primarily *Trypanosoma brucei* as a model, several groups have begun to elucidate the basic regulatory mechanisms and to define the cellular factors controlling transcription, processing, degradation and translation of mRNAs in trypanosomatids. This chapter describes studies that have been focused on a subset of genes that are differentially expressed during the life cycle of *T. brucei, T. cruzi* and few species of *Leishmania.* Although a predominance of regulatory pathways acting at a post-transcriptional level is found for most genes from all three parasites, it is also evident that the regulatory strategies chosen by different trypanosomatid species are not similar. Because of their complex and diversified gene regulatory machinery, *T. brucei, T. cruzi* and *Leishmania* spp. are able to respond rapidly to the drastic environmental changes they face during their life cycle, particularly when they move between their different hosts.

Keywords: Epigenetics, Gene expression, Polyadenylation, Post-transcription, RNA polymerase, RNA promoter, *Trans*-splicing, Transcription, *U*ntranslated region, VSG.

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INTRODUCTION

Trypanosomatids have Unusual Mechanisms of Gene Expression

Within the *Trypanosomatidae* family, the genera *Trypanosoma* and *Leishmania* consist of several species of uniflagellated protozoa parasites that are the causative agents of several tropical diseases, such as sleeping sickness (African trypanosomiasis), caused by *Trypanosoma brucei gambiensis* and *Trypanosoma brucei rhodesiense*; Chagas disease (American trypanosomiasis), caused by *Trypanosoma cruzi*; and different forms of leishmaniasis, caused by various species of *Leishmania*. According to recent World Health Organization surveys, it is estimated that approximately 20 million people are infected with either one of these parasites and 550 million people in the developing world are at risk of contracting leishmaniasis, Chagas disease or sleeping sickness (The World Health Report, 2014, http://www.who.ch). These digenetic parasites have distinct life cycles that present multiple differentiation forms alternating between various types of invertebrate and vertebrate hosts. Details about *Trypanosoma* and *Leishmania* life cycles and their hosts are described in Chapter 1. Therefore, the parasites must rely on multiple regulatory mechanisms to rapidly respond to the drastic environmental changes they face every time they move between their different hosts. *T. brucei, T. cruzi* and a few species of *Leishmania* are the model systems used in the studies herein described. These species belong to an ancient group of unicellular eukaryotes, which, although we tend to consider as closely related organisms, are in fact highly divergent evolutionarily [1]. Because there are still no effective drugs to treat or vaccines to prevent diseases caused by the trypanosomatids, there continues to be an urgent need for basic research that can reveal new molecular targets and parasite-specific pathways with the potential of creating new prophylactic tools and more efficient drug therapies. Details about vaccine candidates against all Tritryps and new therapeutic tools are available, respectively, on Chapters 10 and 9.

In addition to their medical relevance, trypanosomatids have been used as models for gene expression studies because of many eccentricities found in their biology. Unlike most eukaryotic genes, which are transcribed into pre-mRNAs containing exons (coding sequences) and introns (mostly non-coding sequences), trypa-

nosome protein-coding genes are intronless and are transcribed into polycistronic pre-mRNAs that are processed into mature mRNAs through "*trans*-splicing" reactions (reviewed in [2]). RNA polymerase II (RNA pol II) is the enzyme responsible for the transcription of protein-coding genes in eukaryotes. In *T. brucei*, however, two groups of protein-coding genes are transcribed by RNA polymerase I (RNA pol I), which, in most eukaryotes, exclusively transcribes ribosomal RNA genes. The co-transcriptional capping of RNAs that occurs in most eukaryotes is mediated by interactions between RNA pol II and capping enzymes. Because in trypanosomatids the addition of an m7G (cap) to the 5′ end of the primary transcript occurs during *trans*-splicing, RNA pol II-dependent transcription of protein-coding genes can be bypassed in trypanosomes. RNA pol I-dependent transcription occurs not only for a few endogenous trypanosome protein-coding genes but also for most foreign genes that can be expressed in the three groups of parasites using vectors containing RNA polymerase I promoters [3]. Indeed, except for SL genes (see below), no RNA pol II promoters have been identified in trypanosomatids. In addition to these differences regarding transcription of nuclear genes, other bizarre aspects of trypanosome biology include the unusual structure of the mitochondrial genome, called kinetoplast DNA (kDNA), and the extensive post-transcriptional modification of mito-chondrial RNAs known as RNA editing, which is required to produce functional mitochondrial mRNAs [4].

Direct evidence for polycistronic transcription at a genomic level was derived from the first complete sequence analysis of chromosome 1 from *L. major*, which contains 79 genes, 29 of them encoded on one DNA strand and the remaining 50 on the opposite strand [5]. Nuclear run-on assays with strand-specific probes showed that, although a low level of nonspecific transcription likely takes place over the entire chromosome, RNA pol II-mediated transcription initiates within the strand-switch region (between the two divergent polycistronic transcription units, or PTUs) and proceeds bidirectionally towards the telomeres [6] (Fig. **1**). Sequencing of the complete *T. brucei*, *T. cruzi* and *L. major* genomes, known as the TriTryp genomes [7 - 9], revealed similar architectures in all three parasite genomes. More recent studies based on genome-wide RNA-seq analyses of the *T. brucei* genome confirmed that transcription initiation is not restricted to regions at

CHAPTER 7

Virulence Factors and Immune Evasion in *Leishmania* spp.

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Abstract: Protozoan parasites of the genus *Leishmania* cause a group of diseases, known as leishmaniasis, affecting humans and also household pets, mainly canids. In the human host, different pathological outcomes ranging from self-healing cutaneous lesions to systemic visceral leishmaniasis are produced by these parasites; these diseases affect millions of people worldwide. Similar to a virus, bacteria and other parasites, *Leishmania* need to evade immune destruction with the aim of completing their life cycle in their mammalian hosts. Moreover, the long co-evolutionary history between parasites of the genus *Leishmania* and their hosts for several millions of years has led to a balanced relationship. To avoid the powerful immune system of mammals, the parasite has developed a set of sophisticated mechanisms to persist, replicate, and spread.

Keywords: Complement system, Exosomes, Glycosylinositolphospholipids (GIPLs), IFN-γ, Immune response, Lipophosphoglycan (LPG), Macrophage, Neutrophil, Phagolysosome, Virulence factor.

INTRODUCTION

The diverse clinical manifestations of leishmaniasis, an infectious disease caused by species of the genus *Leishmania*, are dependent both on the virulence of the infecting species and on the immunological status of the host. This disease, in its cutaneous form, has been known and described since antiquity. Thus, human

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representations showing faces afflicted with cutaneous leishmaniasis (CL) have been found in Peru and Ecuador and dated 400-900 AD. The first description of the infectious agent was done by David D. Cuningham in 1885 when analyzing samples from lesions of “Delhi boil” in India. In 1903, independent investigations carried out by William Leishman and Charles Donovan led to the identification of the parasite in the spleen of visceral leishmaniasis (VL) patients. Soon after, Sir Ronald Ross named the parasite *Leishmania donovani* (see [1] and references therein).

Leishmaniasis cases have been reported in 98 countries and three territories [2]. Around 0.2 to 0.4 million and 0.7 to 1.2 million of cases of visceral and cutaneous leishmaniasis, respectively, occur each year. More than 90% of global VL cases occur in India, Bangladesh, Sudan, Ethiopia and Brazil. CL is more widely distributed, but 70 to 75% of cases concentrate in ten countries: Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, North Sudan, Peru and Syria. It is likely that the actual incidence of leishmaniasis is underestimated, due to misdiagnosis and under-declaration. The relevance of this parasitic disease is further stressed out by the rise of *Leishmania*/HIV co-infection in many parts of the world, including European countries where up to 9% of the AIDS patients, before the introduction of HAART therapy, also experienced VL [3].

The long evolutionary history between *Leishmania* and their mammalian hosts has modulated both the strength of the immune response against the parasite and the virulence of the pathogen, leading often to a peaceful cohabitation (asymptomatic infection). Thus, the understanding of this balanced relationship will be useful for understanding disease progression and, therefore, paramount for developing immunotherapies for controlling leishmaniasis.

At present, there is no effective vaccine to prevent leishmaniasis in humans, despite effective vaccination against cutaneous leishmaniasis has been practiced for centuries by a procedure known as leishmanization, which consisted in the deliberate inoculation of virulent organisms from the exudates of active lesions [4]. The major hurdles for developing immunotherapies to either prevent or treat leishmaniasis are both the complexity of the host-pathogen interactions and our limited understanding of the precise immune mechanisms required for controlling

parasite growth without causing disease. Details about *Leishmania* spp. vaccine candidates and prophylactic protocols are available in Chapter 11. Current treatments for leishmaniasis patients rely on chemotherapy, but drug treatment is undermined by unwanted toxicity problems in patients and the appearance of drug-resistant parasites [5].

Table 1. ***Leishmania*** **virulence factors involved in immune evasion.**

Leishmania **molecule**	**Effects on immune response and pathology**	**References**
A2	Associate with visceral tropism	[18]
Cysteine peptidase B (CPB)	Promotes Th2-type responses	[222, 224]
Elongation translation factor-1α (EF-1α)	Blockage of iNOS expression in response to macrophage treatment with IFN-γ	[195]
Fructose-1,6-bisphosphate aldolase	Interferes with the induction of nitric-oxide synthase in response to IFN-γ treatment	[197]
GP63 (leishmanolysin)	Resistance to complement-mediated lysis, inhibition of natural killer cellular functions, resistance to antimicrobial peptides	[39]
Lipophosphoglycan (LPG)	Blocks NADPH oxidase assembly at the phagosome membrane; suppresses IL-12 production by host macrophages	[81, 221]
Glycosylinositolphospholipids (GIPLs)	Inhibition of nitric oxide synthase; inhibit production of TNF-α	[35, 36]
Amastigote proteophosphoglycan (aPPG)	Ectopic activation of complement, avoiding parasite lysis by complement	[34]
Promastigote surface antigen (PSA)	Binding to CR3 of macrophages, protects *Leishmania* from complement-mediated lysis	[49] [48]
Ribosomal protein S3a	Inhibits T-cell proliferation and downregulates secretion of IFN-γ, IL-2 and IL-12	[220]
Leishmania RNA virus	Its presence is associated with metastasizing ability of *L. guyanensis*	[27]

This chapter is focused on characterized virulence factors (Table **1**), either membrane-associated or secreted by *Leishmania* that are involved in subverting host immune responses, favoring the immune evasion and persistence of the parasite. For getting a greater understanding of the evasion mechanisms, a comprehensive overview of our current knowledge on the host immune responses during *Leishmania* infection and the immune cells hosting the parasite is also presented.

CHAPTER 8

Virulence Factors and Immune Evasion in *Trypanosoma cruzi*

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Abstract: Among the pathogens that have developed a variety of strategies to overcome the host immune system, is the causative agent of Chagas disease, *Trypanosoma cruzi*. During a long co-evolution process, the parasite has learned how to live in many different environments, including vertebrate and invertebrate hosts. The parasite has also evolved many invasive strategies, including several different ways to enter the host and also the capacity to target different host tissues. An acute systemic response arises in the host after the rapid parasite colonization, interfering with both innate and adaptive immunity. The capacity of *T. cruzi* to interfere with humoral and cellular immune responses is demonstrated by the expression of different sets of molecules called virulence factors. Among them, the role of antioxidant enzymes, cruzipain, the Tc85/transialidase superfamily, mucins, MASPs, GPI anchors, complement regulatory proteins and others are discussed in this chapter. The expression of parasite-specific virulence factors allows *T. cruzi* to overcome host immunity successfully and also to invade and disseminate in many different mammalian hosts. However, the picture that has emerged suggests that the basis and mechanisms of parasite virulence could be more complex than expected. Different aspects such as parasite genetic diversity, the effects of polyparasitism and the potential effects that vertebrate and invertebrate hosts have on parasite virulence and the outcome of natural or experimental infection by *T. cruzi* should be taken into account in futures studies to understand *T. cruzi* virulence.

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Keywords: Complement regulatory proteins, CRP, GPI anchor, Immune evasion, MASPs, Mucins, Siglec, Small mucin-like gene, Tc85, *Trans*-sialidase.

INTRODUCTION

Trypanosoma cruzi is a kinetoplastid flagellate protozoan and the etiologic agent of Chagas disease. *T. cruzi* has a digenetic life cycle involving an invertebrate and a vertebrate host, including the human species. The parasite probably appeared in the region that originated the American and infected primitive mammals over 150 million years ago [1]. Thus, the wild cycle of *T. cruzi* has existed in nature for millions of years [2] and according to several authors [3, 4], humans made contact with the *T. cruzi* transmission cycle after acquiring sedentary habits that also included the domestication of animals and plants. In this scenario, several pieces of evidence suggest the hypothesis that *T. cruzi* infection is perhaps as antique as man's stay in the American continent. Consistent with this, PCR-positive samples from mummies from pre-Colombian Chile and Peru, were positive for *T. cruzi* infection indicating the occurrence of Chagas disease 9,000 years ago [5, 6]. This suggests that all along these years, *T. cruzi* has performed life cycles in triatomines, humans, domestic and wild mammals. During this long co-evolution process, the parasite has learned how to survive in many different hosts, developing an extensive repertoire of molecules that allows it to infect different triatomine species from the *Reduviidae* family and nearly all the tissues of more than one hundred species of mammals. Another significant issue of *T. cruzi* adaptive biology regards to the many ways that *T. cruzi* use to be transmitted to mammalian hosts. The infectious metacyclic trypomastigote (MT) stage can be transmitted principally by blood transfusion, by contamination of the skin and mucous membranes and by congenital and oral contact. The biological plasticity of *T. cruzi* facilitates the variability, complexity and distinct natural transmission cycles on a temporal and spatial scale. The complexity of its natural transmission cycles shows that over the time, humans may have contacted *T. cruzi* on several opportunities and through many different ways, according to principally how people interacted with the environment [7].

T. cruzi Developmental Cycle

The *T. cruzi* developmental cycle is divided into two different hosts. It can be initiate with a hematophagous triatomine, which during its blood meal could be infected with blood trypomastigotes from the mammalian host. After the triatomine infection, parasite migrates through the digestive tract of the triatomine and differentiate into the replicative, non-infectious form named epimastigote. In the insect gut, epimastigotes multiply by binary fission and migrate to the rectum to differentiate into the non-replicative and infective, MT form, which is excreted with the insect feces. During this transformation process known as metacyclogenesis, parasite develops adaptations in biochemical and morphological ways that allow it to invade nucleated cells and resist the immune attack of the vertebrate host.

The MT excreted by the triatomines penetrates the mucosal of the vertebrate host or the puncture mark at the wound generated by the insect. Inside the vertebrate host, MT can invade different cell types, such as fibroblasts, macrophages and muscle cells [8], and evades the highly oxidative phagocytic environment, starting the mammalian cycle. Upon surviving this hostile environment, MT escapes from the phagolysosomal vacuole and in the cytoplasm transform into amastigotes, which will multiply and converts into blood trypomastigotes [8]. Finally, trypomastigotes reach the blood vessels in order to disseminate the parasite infection in different cells and organs. Details about parasite life cycle and the morphology of different parasite life stage are described in Chapters 1 and 2.

THE MECHANISMS OF *T. CRUZI VIRULENCE*

For parasitologists, virulence is always described as the increase in host lethality resulted by pathogen invasion [9]. Even though, this is explicit and quantifiable, it does not consider several aspects of parasite biology that result in damage to the cells and organs without host death. For clinical microbiologists, virulence is comprehended as injury or sickness to the hostal though it does not explain how this damage is caused and which virulence factors could be involved in this harm [10].

Virulence factors are described as molecules synthesized and expressed by a

CHAPTER 9

Molecular Tools and Strategies for Diagnosis of Chagas Disease and Leishmaniasis

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Abstract: Chagas disease and leishmaniasis, caused by the kinetoplastid protozoans *Trypanosoma cruzi* and *Leishmania* spp., respectively, affect millions of people worldwide, most of them belonging to neglected populations. Diagnostic tests for Chagas disease are employed during epidemiological surveys of vectorial and oral transmission, blood bank screening, analysis of pregnant women and their newborns, and in individual cases. However, the currently available assays need improvement. The different phases of the disease, the transmission mode and the high genetic variability of the parasite increase the difficulties of making diagnostic kits with different markers suitable for the diverse scenarios of *T. cruzi* infection. Different *Leishmania* species cause diverse clinical features and sequelae and require different clinical management. In contrast to Chagas disease diagnosis, molecular diagnosis for leishmaniasis requires not only confirmation of the infection but also the genotyping of complexes, species or subspecies. Precise diagnosis and rapid species identification can facilitate decision-making respect to treatment and follow-up of parasite spread. The aim of this chapter is to summarize the most commonly used molecular tools described to date to detect *T. cruzi* infection and to detect and genotype *Leishmania* spp.

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Keywords: Benznidazole, Chagas disease, HIV co-infection, Internal transcribed spacer, Isoenzymes, Leishmaniasis, Loop-mediated amplification, Molecular diagnosis, Recombinant antigens, Serodiagnosis.

CHAGAS DISEASE

Introduction

Chagas disease (CD), probably "the most neglected of the neglected diseases" is caused by the kinetoplastid parasite *Trypanosoma cruzi* [1]. Despite it has traditionally been treated as an endemic disease in the tropical and subtropicals areas of South and Central America, and Mexico, it is an emerging global concern due to the increasing migration of affected people to non-endemic areas, and the perpetuation of infection through congenital or transfusional transmission [2]. The outcome of the infection is a consequence of pathogen-host interactions modulated by their genetic composition, immunological host response and eco-epidemiological factors.

The acute phase of CD presents variable symptoms which generally decline spontaneously some weeks after infection. Prompt etiologic treatment usually eliminates the parasite during acute CD, although symptoms are only detected in 1-2% of infected individuals. The chronic phase is asymptomatic in around 70% of seropositive persons and 30% manifests digestive and/or cardiac pathologies years or decades later, that can evolve to heart failure and sudden death. *T. cruzi* infected immunosuppressed patients may develop necrotizing inflammatory injury in the central nervous system. Prognostic markers of disease progression remain to be identified [3].

Investment devoted to CD has been insufficient to provide efficient diagnosis and treatment tools for patient management and control programs [4].

Genetic Organization of T. cruzi

T. cruzi populations present a complex structure, consequence of clonal propagation [5, 6] as well as rare events of gene exchange [7]. Different biological, biochemical and molecular studies have demonstrated many genetic

and protein polymorphisms [5, 8, 9]. By different markers, natural populations can be classified into six discrete typing units (DTUs Tc I to Tc VI), which are composed of sets of parasite stocks genetically closer to one another than to any other one. These DTUs are identifiable by specific molecular markers [10], they show different geographical distribution [11], DNA content and gene dosage [12 - 15]. All DTUs can cause CD as they are infective to humans. Different histotropism were reported in patients infected with DTU mixtures [16, 17]. *T. cruzi* genetic diversity should be taken into account when developing diagnostic tests for worldwide use and any new test should be validated with strains representing all DTUs. Indeed, some molecular targets used for recombinant antigen and nucleic acid amplification based diagnoses are polymorphic and present different gene dosage in strains belonging to different DTUs and in some instances in strains from the same DTU.

Current Situation of Chagas Disease Diagnosis

Diagnostic tests may be used during epidemiological surveys, blood bank screening, analysis of pregnant women and individual cases with suspicion of infection, but the available assays need improvement. In particular, no effective tools are available for population screening, the point of care settings or follow-up of anti-parasitic treatment.

The different transmission modes, the disease phases and the high genetic variability of the parasite increase the difficulties of making diagnostic kits with most appropriate markers for the diverse CD epidemiological settings.

The Polymerase Chain Reaction (PCR) has opened new opportunities to detect *T. cruzi* infection and to evaluate trypanocidal chemotherapy [18 - 20]. However, PCR procedures may have highly variable sensitivity, specificity and accuracy, depending on the type and volume of clinical sample, the storage conditions, the DNA purification methods, the parasite genes used as targets, primers and probes, the reagents as well as the thermocycling parameters. The intermittent blood-stream parasitic load is another factor responsible for the PCR clinical sensitivity. False negative results can also occur because of PCR inhibitors that can be co-purified during DNA extraction procedures whereas false positives may arise due

CHAPTER 10

New Chemotherapy Against Trypanosomiasis and Leishmaniasis

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Abstract: Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* affecting mostly the American continent eventually leading to chronic cardiomyopathy or digestive syndromes. Human African Trypanosomiasis is caused by the related parasite *T. brucei*, endemic in the African continent and being characterized by invasion and damage in the central nervous system. Leishmaniasis is caused by a number of species from the genera *Leishmania* and can manifest with different clinical outcomes including skin ulcers and visceral organ damage, being endemic in 88 countries in tropical areas of the globe. Despite having different geographical distribution and unique clinical symptoms, these diseases are all caused by related protozoan parasites from the order Kinetoplastida. Another aspect shared by these diseases is related to the treatment options currently available, unfortunately, all inadequate. Serious problems are toxicity and inefficacy due parasite acquired resistance or lack of natural susceptibility. The population affected by these three diseases does not represent an attractive economic market, reflecting on little pharmaceutical industry interest in developing better chemotherapies. However, in the last decade, the situation has dramatically improved, with the active engagement of philanthropic financial support, national government organizations, research centers, the pharmaceutical industry and the Drugs for Neglected Diseases Initiative (DND*i*). After the development of screening assays, millions of molecules have been tested, and some have reached Clinical Trials stage. A milestone has been set for the year 2020, by the London Declaration: control of Chagas disease and leishmaniasis, and elimination

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of Human African Trypanosomiasis. Advances and progress to achieve these goals are presented in this chapter.

Keywords: African Trypanosomiasis, Chagas Disease, Chemotherapy, Drug development, High-throughput hit selection, Lead optimization, Leishmaniasis, Medicinal chemistry, Neglected Diseases, Pharmacokinetics.

INTRODUCTION

Drugs used today to treat trypanosomiasis (Chagas disease – CD and Human African Trypanosomiasis – HAT, or sleeping sickness) and leishmaniasis (both Visceral Leishmaniasis – VL and Cutaneous Leishmaniasis – CL) are old and not adapted to the field. All have drawbacks, especially toxicity, resistance and inefficacy. The fact that no effective vaccines exist against these diseases (see Chapter 11 for details), supports the urgent need for new and better treatments. Since the affected population does not represent an attractive economic market, the pharmaceutical industry has neglected these diseases for decades [1]. The World Health Organization (WHO) named Neglected Tropical Diseases (NTDs) a group of 17 diseases in a similar situation. In addition to the three caused by kinetoplastid parasites focused in the chapter (Chagas disease, HAT and leishmaniasis), the other diseases are dengue, rabies, trachoma, Buruli ulcer, endemic treponematoses, leprosy, taeniasis/cysticercosis, dracunculiasis (guinea worm disease), echinococcosis/hydatidosis, foodborne trematodes, lymphatic filariasis, onchocerciasis (river blindness), schistosomiasis, and soil-transmitted helminthiases [2]. Despite the significant burden caused by these diseases, for decades not much has been done regarding funding, research, and public health policy. As a group, they significantly increase morbidity and mortality in tropical areas of the globe, representing a substantial economic burden on the affected endemic countries [3, 4]. For three decades starting from the 70's, only 21 drugs out of 1,556 (1.3%) were approved specifically to treat NTDs, despite the fact that NTDs affect 11.4% of the disease burden in the world.

Branding these diseases with a collective name (NTDs) was a wise marketing strategy to raise funds and awareness according to Dr. Hotez [5, 6]. The World Health Organization has recently set ambitious goals to control or eliminate 10 of

the NTDs by the year 2020 and significantly reduce the burden of the other 7 NTDs. A meeting held in London in 2012 brought together representatives from the pharmaceutical industry, donors, and politicians who all agreed on a series of commitments to provide more drugs, research, and funds to achieve the 2020 goals. The document with the mission statements is known as the "London Declaration" [7]. Regarding the diseases caused by kinetoplastid parasites, the goal is to control CD and leishmaniasis and to eliminate HAT by 2020. A roadmap was launched in this meeting to guide this global plan [8].

The drug discovery process, in general, long and expensive (Fig. **1**), is a highly multidisciplinary endeavor associated with high risk, the high attrition rate of candidate compounds and requires significant amounts of resources [9]. These challenges combined with low perspectives of market profitability make these tropical diseases unattractive for pharmaceutical companies [10, 11] unless special incentives are given [12].

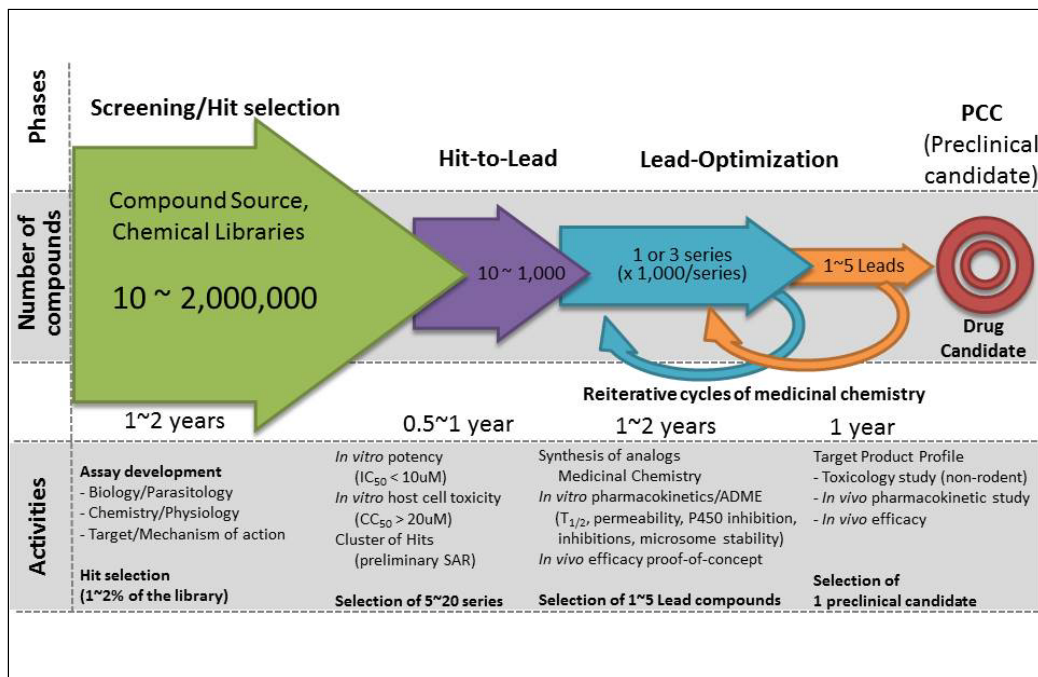

Fig. (1). Drug Discovery Pipeline process with decision-making tools and key decision points.

CHAPTER 11

Recombinant Vaccines Against Pathogenic Trypanosomatids

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Abstract: The *Leishmania* spp, *Trypanosoma cruzi* and *Trypanosoma brucei* spp are the causative agents of tropical infections, and over 20 million people worldwide suffer from these neglected diseases. During the last century, vaccine development has had an undeniable impact on public health and may offer some alternatives for the control of parasitic diseases. Immune protection against experimental infection with these parasites has been studied and many types of immunogens have been used. Use of new technologies has allowed the development of recombinant proteins and DNA-based vaccines against those protozoans, aiming to generate both humoral and cellular protective responses. A large amount of data have been obtained from preclinical model systems which gave us promising results. The main challenge at the present is to translate what has been succeeded in these models into efficient human vaccines. The objective of this review is to summarize the efforts of the science community about the development of recombinant vaccines against trypanosomatids.

Keywords: African trypanosomiasis, Cellular response, Chagas disease, DNA vaccine, Humoral response, Immunotherapy, *Leishmania* spp, Leishmaniasis, *Trypanosoma brucei*, *Trypanosoma cruzi*.

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INTRODUCTION

Trypanosomatids are unicellular protozoans and etiologic agents of infectious diseases in humans as well as livestock. *Leishmania* spp, *Trypanosoma brucei* and *Trypanosoma cruzi* are parasites of medical and economic importance since they are responsible for diseases that cause numerous infections in mammals. In human health, over 20 million people are estimated to be affected, primarily in developing and under-developed countries. The complete genome sequences of *T. brucei* [1], *T. cruzi* [2], and *L. major* [3], known as the TriTryp genome, constitutes an important advance in the study of these parasites. However, human treatment of the diseases is limited to drugs that frequently have adverse side effects, and to date, no suitable vaccines are available [4].

First Generation Prophylactic Vaccines

In general, prophylactic vaccines can be grouped into 3 categories: containing live attenuated microbes, containing killed microbes and containing microbial subunits. In the first group, attenuated agents are obtained by selection of mutants under unfavorable conditions; in the second, inactivated vaccines are composed of parts of the microorganism (carbohydrates and proteins), generally obtained from the surface of the killed pathogens. In both cases, the objective is the induction of antibodies and to evoke cellular responses. The latter group is composed of vaccine formulations containing one or a few components purified from a microorganism, frequently delivered with an adjuvant and administered employing a prime/boost strategy [5].

The First generation group of vaccines, including live attenuated or killed microbes in their formulation, was widely used and allowed a huge advance in the combat of parasitic diseases. Currently, however, there are several inconveniences associated with these vaccines. They frequently have a limited shelf-life, require chilling or freezing, and their production is cumbersome and time-consuming. For live vaccines, moreover, there is the risk of pathogenicity reversion of the attenuated vaccine strains, and/or changes in the parasite population that can lead to reduced protection. For killed or inactivated vaccines, the cellular immune responses are frequently poor.

Second Generation Prophylactic Vaccines

Subunit vaccines, when formulated with purified (native or recombinant) antigens, are considered a Second generation group of vaccines. Use of new technologies has allowed different cells types (bacteria, yeast, animal and insect cells) to be used as tools for the production of recombinant antigenic proteins. These formulations have several distinct advantages over the native proteins, which include easier production, possibility of manipulation using genetic engineering techniques, superior stability, much safer and less expensive. In this scenario, new information about innate immunity processes, of the different antibody functions, and of both $CD4^+$ and $CD8^+$ T cells functions in response to vaccination, is essential for antigen designs [5]. However, the efficient production, purification and implementation of recombinant proteins for vaccine development can be harder than it appears to be.

Different systems have been employed to produce recombinant proteins for experimental use or biotechnological applications. They include chemical peptide synthesis, microorganisms, animal cells, plant cells, transgenic plants and transgenic animals (reviewed in [6]). Microorganisms, such as bacteria, were the first to be used to produce recombinant proteins. This approach was highly efficient for some proteins, mainly produced in *E. coli* and was used in a broad spectrum of applications, including vaccine development. However, bacteria cannot fold a number of proteins properly (as they are unable of performing adequate post-translational modifications) or can form inclusion bodies precluding an easy purification. In addition, some proteins are toxic for bacteria and cannot be prepared in this way. An alternative is the use of yeast as *Pichia pastoris*, which may be easily transformed and is capable of producing folded proteins and exporting them, facilitating the subsequent purification. The negative point is that yeast often produces a limited amount of recombinant proteins, which are not glycosylated or unduly glycosylated [6]. Still, yeast is the currently preferred system for the production of many recombinant proteins.

Concerning animal and plant as bioreactors, most of the recombinant proteins used as pharmaceuticals have been prepared from animal cells. However, this system is costly and poorly flexible compared to plant cells, in which the yield of

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