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New serological tools for improved diagnosis of human tegumentary leishmaniasis

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ABSTRACT

Human tegumentary leishmaniasis (HTL), characterized by skin ulcers that may spread and cause dreadful and massive tissue destruction of the nose and mouth, is considered a neglected tropical disease, and it is a serious threat to global health due to its continuous expansion, favored by the lifecycle of its causative organism that is maintained in domestic animal reservoirs and anthropophilic sand fly species. Serodiagnosis of HTL is a great challenge due to many biological factors, including hampered specificity and/or sensitivity. This investigation addresses the unmet need for new diagnostic markers of HTL, and describes a simple platform to improve the serodiagnosis. A constrained conformational phage display random peptide library combined with a magnetic microsphere-based subtraction strategy was used to identify ligands with potential diagnostic applications. Six clones were selected against IgG antibodies from HTL patients, characterized by sequencing and confirmed by a phage-ELISA using sera from patients developing visceral leishmaniasis (n = 20), Chagas disease (n = 10), mucosal (n = 30) and cutaneous (n = 20) leishmaniasis; as well as from healthy subjects living in endemic (n = 20) and non-endemic (n = 30) areas of leishmaniasis. A wild-type M13-phage clone and a soluble *Leishmania* antigenic extract were used as negative and positive controls, respectively. Three clones reached 100% sensitivity and specificity, without any cross-reactivity with sera from patients with leishmaniasis-related diseases. Briefly, we describe for the first time a set of serological markers based on three immunodominant mimotopes that showed 100% accuracy, and that could be used in a phage-ELISA assay for the HTL serodiagnosis.

1. Introduction

The human tegumentary leishmaniasis (HTL), caused mostly by *Leishmania* (*Viannia*) *braziliensis* and *Leishmania amazonensis* species, leads to a broad spectrum of clinical manifestations attributed to intraspecific variability of the parasites (Oliveira et al., 2013). The expansion of metropolitan areas associated with the parasite's lifecycle, maintained in domestic animal reservoirs and anthropophilic sand fly species (Grimaldi et al., 1989; Grimaldi and Tesh, 1993), presents a significant threat to global health due to its expanding dissemination worldwide. Nearly 1.5 million cases of HTL have been registered annually (Alvar et al., 2012), and the majority present single or few skin ulcers, but the extreme scarceness of parasites within lesions does not correlate with disease severity and resistance to antimonial therapy (Da-Cruz et al., 2002). Clinical manifestations of disease are classified as cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL) and mucosal leishmaniasis (ML) (Reithinger et al., 2007; World Health O, 2010), but only 3 to 5% of infected patients will develop a severe disease (DCL or ML), which produces a destructive secondary mucosal lesion in the nose and mouth with extensive disfiguration and pain (Da-Cruz et al., 2002). Severe manifestation of CL is associated with a polarized

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Th2, when compared to a Th1 response found in mild manifestations of the disease (Gaafar et al., 1995). Therefore, the T-cell-mediated immune response plays a pivotal role either for cure or aggravation of the disease (Da-Cruz et al., 2002).

HTL diagnosis is currently performed by a combination of methods and clinical symptoms; therefore, there is an urgent need for more precise, simple and fast diagnosis approach (Vega-Lopez, 2003; Goto and Lindoso, 2010). Currently, diagnosis relies on visualizing parasites in tissue or by culture, serology or detection of parasite DNA (Murray et al., 2005). The standard method for laboratory diagnosis of CL and ML is microscopy of Giemsa-stained or hematoxylin-eosin-stained scrapings, aspirate samples, or biopsy samples of skin ulcers or mucosal lesions (Murray et al., 2005), but it presents low sensitivity due to the scarceness of parasites within lesions (Da-Cruz et al., 2002). Another method used is the Montenegro skin test (MST), which measures a delayed-type hypersensitivity (DTH) reaction to an intradermal injection of leishmanial antigens (Manson-Bahr, 1961); however, it requires standardization, trained technician to assure consistent interpretation of results, quality and concentration of antigens injected, as well as it depends of the physiological status of the patients (Antonio et al., 2014).

Besides those complicating factors, the MST still cannot distinguish between patients with acute symptoms from those cured or under treatment (Viana et al., 2011). DNA detection by PCR technique has been described for CL diagnosis, but is not available for patients' diagnostic testing and still requires extensive standardization (Deborggraeve et al., 2008; de Oliveira et al., 2003; Oliveira et al., 2011). Serological tests are employed for HTL diagnosis, and are the most advantageous for being minimally invasive and easy to perform, but lack specificity since HTL-infected patients may present low antibody titers, and yet non-infected individuals living in endemic areas of disease can present false-positive results (Malchiodi et al., 1994; Souza et al., 2013; Duarte et al., 2015). Additionally, cross-reactions are also registered in patients infected by other Trypanosomatidae, such as *Trypanosoma cruzi* or *Leishmania infantum* (Malchiodi et al., 1994; Celeste et al., 2004; Menezes-Souza et al., 2015). The lack of accurate diagnosis is one of the most important factors that have led to the expansion of HTL infection, not only affecting global health, but also worsening poverty in low-income countries due to the greater morbidity. Therefore, improved HTL serodiagnosis is mandatory, and the identification of new antigens must aim the development of more sensitive and specific serological assays.

Currently, advanced proteomic strategies to obtain novel biomarkers have relied on subtractive selection cycles within recombinant libraries containing a very large repertoire of random peptides expressed in the capsid surface of filamentous bacteriophage, also called phage display. Peptide ligands are usually identified and affinity-enriched by cycles of bio-selection against a specific target, and selected peptide sequences that mimic epitopes (mimotopes) of native antigens are further characterized by DNA sequencing and validated by immunoassays (Parmley and Smith, 1988). Phage display has successfully developed antigens for improved diagnosis of malaria (Greenwood et al., 1991; Monette et al., 2001), toxoplasmosis (Beghetto et al., 2003; Cunha-Junior et al., 2010), hepatitis (Larralde et al., 2007), neurocysticercosis (Manhani et al., 2011), strongyloidiasis (Feliciano et al., 2014), Chagas' disease (Pitcovsky et al., 2001) and canine VL (Costa et al., 2014).

Recently, our group have focused on the development of robust diagnostic platforms using highly specific and sensitive markers for visceral leishmaniasis (VL) selected by phage display (Costa et al., 2014; Goulart et al., 2010); but HTL serodiagnosis is still a significant challenge. In the present study, we have performed a stringent subtractive selection in a phage display library expressing constrained (cyclic) peptides, and the selection strategy was further improved by using a magnetic microsphere support and serum samples from CL and ML patients, which led us to the identification of six new mimotopes that were explored as novel diagnostic antigens for the HTL serodiagnosis using a very phage-ELISA immunoassay.

2. Materials and methods

2.1. Sera samples

This study was conducted according to the Declaration of Helsinki principles, and it was approved by Ethics Committee from Federal University of Minas Gerais (protocol number CAAE–323431 14.9.0000.5149), Belo Horizonte, MG, Brazil. All patients received an individual copy of the study policy, which was reviewed by an independent person, and all participants gave their consent form in Portuguese, before collection of their blood sample. Serum samples of patients with confirmed diagnosis of HTL were classified as CL ($n = 20$; including 14 males and 6 females, with ages ranging from 30 to 56 years) or ML ($n = 30$; including 19 males and 11 females, with ages ranging from 22 to 63 years) and, in both cases, they were collected in an endemic area of leishmaniasis (Belo Horizonte, Minas Gerais, Brazil). The diagnosis was confirmed by clinical evaluation of lesions, which were compatible with either CL or ML, as well as by direct demonstration of the parasites in Giemsa-stained smears of biopsies of skin (CL) or mucosal fragments (ML) of patients. Analyses from two biopsy samples collected from each patient were submitted to a conventional PCR technique, and they presented positive results for *L. braziliensis* kDNA. None of the patients had been previously treated with anti-leishmanial drugs, before samples collection. Serum samples were also obtained from healthy individuals living in an endemic ($n = 20$, including 12 males and 8 females, with ages ranging from 17 to 42 years, Belo Horizonte, MG, Brazil) or non-endemic ($n = 30$, including 21 males and 9 females, with ages ranging from 15 to 48 years; Poços de Caldas, MG, Brazil) area of leishmaniasis, and they were used as negative controls. Healthy subjects were clinically evaluated and did not present any clinical signs or suspect of leishmaniasis. Serum samples from Chagas disease patients ($n = 10$, including 7 males and 3 females, with ages ranging from 24 to 58 years) and from patients developing VL ($n = 20$, including 13 males and 7 females, with ages ranging from 18 to 62 years) were used to test cross-reactivity. All sera were collected by venipuncture of medial vein in tubes without anticoagulant, and were kept at 37 °C by 15 min, when they were centrifuged at 4000 $\times g$ for 15 min, and samples were separated and kept at −80 °C, until use.

2.2. Soluble antigenic extract

L. braziliensis (MHOM/BR/1975/M2904) strain was used. Stationary-phase promastigotes of *L. braziliensis* were grown at 24 °C in Schneider's medium (Sigma, St. Louis, MO, USA), which was supplemented with 20% inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. The soluble antigenic extract (SLA) of the parasites was prepared as described (Coelho et al., 2003).

2.3. Phage display selection strategy against IgG antibodies from patients and controls

To perform the affinity selection of phage libraries, IgG antibodies were purified from sera of healthy subjects, as well as from patients developing Chagas disease or HTL (CL and ML), and molecules were coupled to magnetic microspheres (beads) conjugated to protein G (Dynabeads, Invitrogen), as previously described (Greenwood et al., 1991; da Silva et al., 2010). Briefly, 1×10^{10} microspheres were washed 3 times in 1 mL of 0.1 M MES buffer pH 5.0, and pools of sera were added to them and prepared individually (270 μL of a pool of sera of healthy subjects, 240 μL of a pool of sera of Chagas disease patients, 300 μL of a pool of sera of ML patients and 300 μL of a pool of sera of CL patients). Preparations of conjugates were brought to a final volume of 600 μL, submitted to incubation (40 min) under constant agitation, at room temperature, and IgG/microspheres complexes were washed 3 times with 1 mL of 0.1 M MES buffer pH 5.0, to remove the non-conjugated

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