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PROTOZOOLOGY - ORIGINAL PAPER

Biological evaluation of mimetic peptides as active molecules for a new and simple skin test in an animal model

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Abstract

A skin test is a widely used tool in diagnostic evaluations to investigate cutaneous leishmaniases (CL). The actual antigen (Montenegro skin test [MST] antigen) presents some difficulties that pertain to its manufacturing and validation. To contribute to overcoming this problem, we propose the application of new-generation molecules that are based on skin antigen tests. These antigens were obtained through biotechnology pathways by manufacturing synthetic mimetic peptides. Three peptides, which were selected by phage display, were tested as skin test antigens in an animal model (*Cavia porcellus*) that was immunized with *Leishmania amazonensis* or *Leishmania braziliensis*. The peptide antigens, individually (PA1, PA2, PA3) or in a mix (PAMix), promoted induration reactions at 48 and 72 h after the test was performed. The indurations varied from 0.5 to 0.7 cm. In the animals immunized with *L. amazonensis*, the PA3 antigen showed better results than the standard MST antigen. In animals immunized with *L. braziliensis*, two peptide antigens (PA2 and PAMix) promoted induration reactions for a longer period of time than the standard MST antigen. These results validate our hypothesis that peptides could be used as antigens in skin tests and may replace the current antigen for CL diagnosis.

Keywords Phage display · Synthetic peptides · Diagnostic · Neglected disease

Introduction

In recent years, the development of biomolecules has attracted the attention of research groups that aim to produce biotechnological supplies. These biomolecules could be applied as sources of pharmaceuticals or even used for diagnoses (Brigido and Maranhão 2002; Bazan et al. 2012; de Moura et al. 2016). As the primary and final product of genetic information pathways, proteins are the most abundant biomolecules in cells and are essential for the biological process. The use of a full-length protein as an antigen can provide multiple epitopes throughout the sequence for antibody recognition and binding. However, these molecules enhance the probability of nonspecific cross-reactivity with homologous epitopes from other proteins (Hancock and O'Reilly 2005; Nagill and Kaur 2011). Once the immunogenic sites of proteins from pathogens are encoded by a small, specific sequence of amino acids (epitopes), these peptides can represent an alternative to recombinant proteins, thereby avoiding the limitations of nonspecific cross-reactivity.

The peptide sequence that triggers the immune response can be identified and chemically synthesized for application as an immunogen in vaccines and within diagnostic approaches (WHO 1997; Brigido and Maranhão 2002; Alban et al. 2014). Synthetic peptides represent well-defined epitopes with highly conserved, unique regions and active sites that can raise the specificity of the antibody ligation (WHO 1997). In a research related to new vaccines and diagnosis markers, synthetic peptides represent a promising approach for developing antigens with high specificity and sensitivity. Some studies have already described the ability of phage-displayed peptides to induce protection against bacteria,

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viruses, and parasites (WHO 1997; Chen et al. 2001; Brigido and Maranhão 2002; Wu et al. 2006; Wang et al. 2007; Alban et al. 2013). In this way, our group has successfully used a phage-display technique to identify the mimotope peptides of different pathogens (Capelli-Peixoto et al. 2011; Alban et al. 2013; Fogaça et al. 2014; Thomaz-Soccol et al. 2015; Link et al. 2017). A study performed by Link et al. (2017) selected three peptides by phage display that use immunoglobulins against *L. braziliensis*. These molecules presented 92 to 100% ability for CL diagnosis when applying an immunoassay test (Link et al. 2017). The sequences of these three peptides were analyzed in a data bank and show homology with *Leishmania* glycoprotein (GP63), lipophosphoglycan (LPG), and other hypothetical *Leishmania* proteins. These results raised the question of whether these peptides can be used for research into the immune response in a cutaneous leishmaniases (CL) diagnosis.

The skin test is one of the most frequently used complementary methodologies in CL diagnosis due to its sensitivity and specificity. Further, it is an easy method to read, is inexpensive, may be easily to transport and can be taken to places that are difficult to access, does not require sophisticated equipment, and can be realized in loco by a healthcare worker. This test is an immunological approach that is based on a delayed-type hypersensitivity (DTH) response in which an in vivo manifestation can be measured and semi quantified by the skin test. The current formulation of the skin test antigen consists of a suspension of promastigote forms of *Leishmania* sp. that varies according to the etiological agent from each region (Skraba et al. 2015). The lack of a standard antigen represents the main problem of this test, which makes its interpretation difficult. Furthermore, the production of the antigen is time consuming and difficult to scale up (Sadeghian et al. 2013; de Vries et al. 2015). These difficulties increase the need to investigate new alternatives for skin test antigens with high specificity and sensitivity and that are capable of promoting a strong DTH response.

The in vivo manifestation of a DTH response can be evaluated in animal models such as *Cavia porcellus*. This animal species is a well-established biological model for the skin test (Malaghini et al. 2011; Alban et al. 2013). In a recent study, Guedes et al. (2017) successfully validated *C. porcellus* as an animal model for the quality control process of the Montenegro skin test (MST) antigen production. This result opens doors to testing new skin test antigens for CL diagnosis in an animal model capable of reproducing a DTH response.

According to these outcomes and with the results that were previously obtained by our group (mimetic peptide production and animal species validation), the present work aimed to confirm the hypothesis that using these antigens in the skin test promotes a DTH reaction in *C. porcellus* immunized with *L. amazonensis* or *L. braziliensis*.

Material and methods

Experimental procedures

In this study, the use of mimetic peptides as skin test antigens in the animal model *C. porcellus* (guinea pigs) was evaluated. The choice of the animal model was based on the previous work of our group (Guedes et al. 2017). The present study was conducted in four phases: (1) peptide chemical synthesis, (2) peptide antigen formulation, (3) *C. porcellus* immunization with *L. amazonensis* or *L. braziliensis*, and (4) the evaluation of peptides as antigens for a skin test against CL (Fig. 1).

Synthesis of mimetic peptides

The three peptides selected for this study were chemically synthesized by the Fmoc strategy (9-fluorenylmethyloxycarbonyl) when using a peptide synthesis protocol in the solid phase (resin as insoluble solid support), which involves special amino acids for in vitro synthesis. The protocol was performed according to the approach outlined by Merrifield (1969) that uses the MultiPep RS automated peptide synthesizer (Intavis Bioanalytical Instruments, Nattermannallee, Germany). Briefly, the peptides' amino acid C-terminals were fixed to the resin and lengthened by successive additions of residues of the C-terminals to the N-terminal portions. The amino groups of these amino acids were protected by the Fmoc group, and each side chain was also safeguarded by a protective group to prevent undesired reactions. The coupling and deprotection cycles (removal of the Fmoc grouping by the presence of 20% piperidine) were performed until all amino acids within the synthesized peptides were coupled. Upon completion of the last cycle, the peptides were removed from the resin by cleavage. The peptide solutions were filtered and precipitated with cold ethyl ether, yielding the peptides. After centrifugation, the ether was discarded. The peptides were lyophilized, weighed, dissolved in ultrapure water, and stored at − 20 °C at a concentration of 1 mg/mL. The peptides' amino acid sequences were confirmed by mass spectrometry using an Autoflex spectrometer (Bruker Daltonics, Bremen, Germany) and FlexAnalysis software (Bruker Daltonics, Bremen, Germany).

Peptide antigen formulation

Peptides were individually conjugated to the carrier protein, bovine serum albumin (BSA) by glutaraldehyde per the work of Harlow and Lane (1988). The carrier proteins (0.03 μmol) were dissolved in phosphate buffer solution (PBS) (pH 7.2) and added to the peptide solution (0.6 μmol), which was also dissolved in PBS. Equal volumes of 0.2% glutaraldehyde in PBS were slowly added to the solution peptide/carrier proteins. The addition of glutaraldehyde occurred under constant

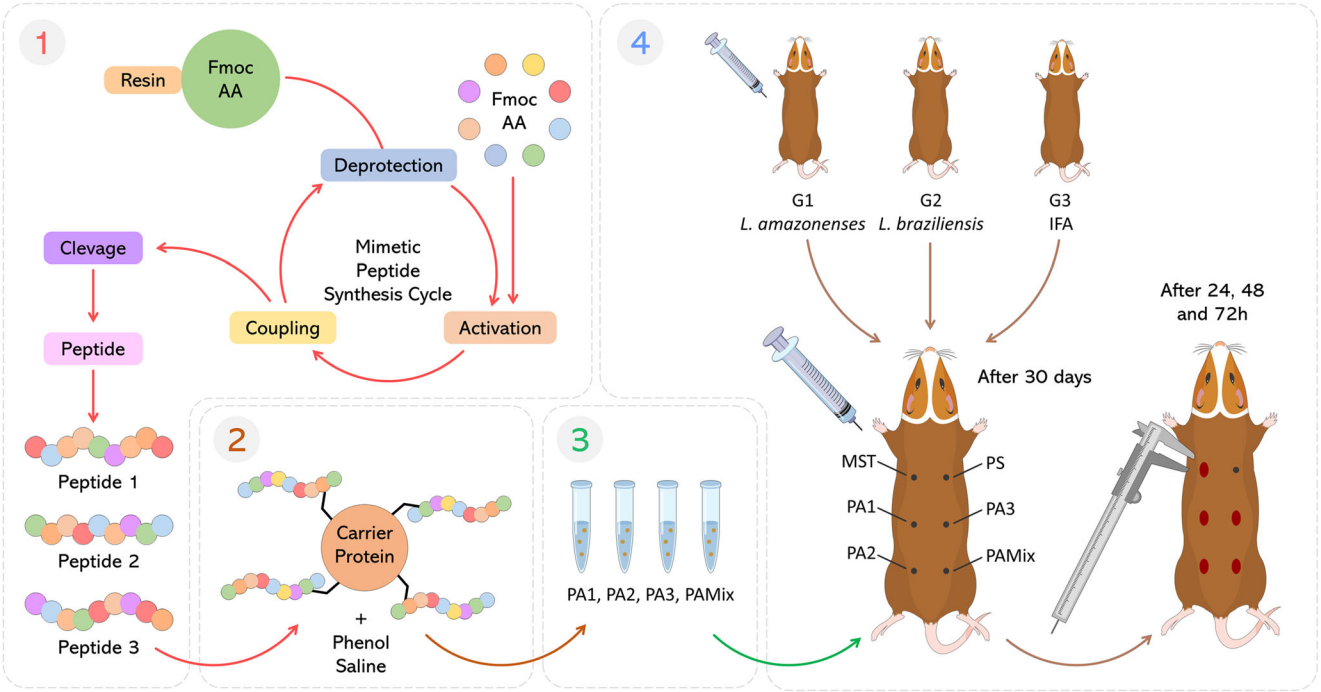

Fig. 1 Experimental procedure steps: (1) peptide chemical synthesis, (2) peptides conjugation to a carrier protein, (3) peptide antigen formulation, and (4) *C. porcellus* immunization and evaluation of the mimetic peptides as antigens for skin test

agitation for 1 h at room temperature. Then, a solution of glycine 1 M in PBS was added to a final concentration of 200 mM. The solution was incubated for 1 h under agitation and at room temperature. Aliquots were stored at − 20 °C.

To formulate the antigens, 80 μg of each lyophilized peptide was diluted in 2 mL of phenol saline solution to a final concentration of 4 μg/0.1 mL. This resulted in four different peptide antigen formulations as follows: PA1 (Pep 1 conjugated to BSA + phenol saline), PA2 (Pep 2 conjugated to BSA + phenol saline), PA3 (Pep 3 conjugated to BSA + phenol saline), and PAMix (each conjugated peptide + phenol saline).

Animal model immunization with *L. amazonensis* or *L. braziliensis*

The following equation was used for the animal number calculation: sample size = $2 \times SD^2 \times (Z^{\alpha/2} + Z^{\beta})^2 / d^2$ (Charan and Kantharia 2013), where standard deviation was = from previous studies; $Z^{\alpha/2} = 1.96$ with an error type 1 of 5%; $Z^{\beta} = Z_{0.20} = 0.842$ at 80% power; d = effect size = difference between mean values. According to this formula and based on a study with human patients (for details, see Skraba et al. 2015), the sample size for this antigen screening study was 4.58, where SD = 0.591 cm corresponds to the standard deviation of the mean value from the positive group from the study with human patients and $d = 1.119$ cm corresponds to the difference between the mean value of the positive and the negative groups from the study with human patients (Skraba et al. 2015). Therefore, we assume five animals per group. Besides that, in in vivo experimental tests, it is important to respect the 3R rule and use the minimum number of animals (Zurlo et al. 1996).

Fifteen adult male *C. porcellus* were immunized with soluble antigens of *L. amazonensis* (MHOM/BR/73/M2269) or *L. braziliensis* (MHOM/BR/94/M2903). The soluble antigens of *L. braziliensis* or *L. amazonensis* were prepared as described by Szargiki et al. (2009), which consisted in a protein extraction of promastigotes diluted in sterile water, followed by the incorporation of incomplete Freund's adjuvant (IFA). The animals were divided into three groups as follows: G1 (immunized with *L. amazonensis* soluble antigen), G2 (immunized with *L. braziliensis* soluble antigen), and G3 (inoculated with IFA and a saline solution; negative control group). The animals were intramuscularly inoculated with 1 mL (4 mg/mL) of a soluble antigen or IFA.

Evaluation of peptides as antigens for skin test

After 30 days of immunization, the animals were skin tested with four new peptide antigens (PA1, PA2, PA3, and PAMix), the current antigen for the CL skin test MST (CPPI—lot 01/12—registration number: 80151040004), and with phenol saline solution (vehicle). One day before the skin test, the animals' dorsal areas were depilated using a chemical depilatory product (Veet®) according the following steps: (1) the depilate cream was spread in thick layers that were enough to completely cover the fur; (2) after 5 min, a wet gauze was used to verify whether the fur could be removed. If the fur was not easily removed, the product was allowed to act for a

longer time that did not exceed a total of 10 min; (3) once the fur could be easily removed, it was wiped away on each animal with wet gauze; (4) the animals' skin was washed with plenty of warm water to completely remove the cream; (5) the animals' skin was dried with soft towels. For the skin test, six inoculation points were delimited (Fig. 1). Each point was intradermally inoculated with 0.1 mL of one of each the five antigens and the vehicle. The antigens were inoculated in a concentration of 4 μg/0.1 mL. The readouts of the skin test reactions were performed at 24, 48, and 72 h after the intradermal inoculation by measuring the diameter of the indurations with a caliper.

Statistical analysis

A Shapiro-Wilk test for the normal condition of variable evaluation and an ANOVA analysis with a Tukey posttest were performed to analyze the significant difference among groups. All statistical analyses were performed using R software, version 3.4.0, assuming a significance level of 5% (R Development Core Team 2011).

Results

Peptides characterization

The three peptide sequences and their molecular weights are shown in Table 1. The molecular weights varied from 1931.059 to 2021.203 Da. For more details about the peptides' identification, see Link et al. (2017).

Biological evaluation of peptides

The average diameters of induration reactions in *C. porcellus* immunized with *L. amazonensis* show that the three antigen peptides (PA1, PA2, and PA3) presented induration reactions, even when injected individually or in the mix (PAMix) (Table 2). The induration reaction of PA1 varied from 0.47 cm at 48 h after the intradermal test to 0.46 cm after 72 h. PA2 presented diameters ranging from 0.54 cm (48 h) to 0.53 cm (72 h). For PA3, the average diameters were 0.61 cm (48 h) and 0.72 cm (72 h). PAMix promoted induration reactions of 0.54 cm (48 h) and 0.58 cm (72 h). The induration result from the phenol saline injection was 0 cm both times that the test was performed. When compared to the MST antigen, the new antigens (PA2, PA3, and PAMix) showed an average induration reaction that was longer and ≥ 0.5 cm, even after 48 through 72 h.

The statistical analysis showed that after 48 h, the difference between the induration reaction promoted by the standard MST antigen and peptide antigens (PA1, PA2, and PA3) produced by our group was not significant (p value = 0.16); however, after 72 h, the induration reaction promoted by PA3 was significantly different from the standard MST antigen ($p = 0.02$) (Fig. 2).

The average diameters of induration reactions in *C. porcellus* immunized with *L. braziliensis* show that PA1, PA2, and PA3 presented induration reactions, even when injected individually or in the mix (PAMix) (Table 3). PA1 promoted induration reactions that varied from 0.55 cm at 48 h after an intradermal test to 0.57 cm after 72 h. For PA2, the average diameters were 0.56 cm (48 h) and 0.71 cm (72 h). PA3 presented diameters ranging from 0.6 cm (48 h) to 0.67 cm (72 h). The induration reactions of PAMix were 0.59 cm (48 h) and 0.73 cm (72 h). The induration result from the phenol saline injection was 0 cm for both test times. When compared to the MST antigen, PA1, PA2, PA3, and PAMix showed an average induration reaction of ≥ 0.5 cm, even after 48 h through 72 h.

The statistical analysis showed that after 48 h, the difference between the induration reactions promoted by a standard MST and the peptide antigens (PA1, PA2, and PA3) produced by our group were not significant ($p = 0.49$), although after 72 h, the induration reactions promoted by PA2 and PAMix were significant from standard MST ($p = 0.01$) (Fig. 3).

In the negative control group (G3), in which *C. porcellus* were injected with IFA and phenol saline, an induration reaction was not observed in the inoculation sites for the standard MST antigen, PA1, PA2, PA3, or PAMix.

In this work, the skin test response promoted by peptides varied from 0.46 (PA1) to 0.61 cm (PA3) at 48 h after the skin test in *C. porcellus* immunized with *L. amazonensis*. In *C. porcellus* immunized with *L. braziliensis*, the indurations after 48 h varied from 0.55 (PA1) to 0.60 cm (PA3). The result of the statistical analysis of skin test responses to peptide antigens was equal to the result shown by the MST antigen in the group immunized with *L. amazonensis* for the skin test after 48 h; the difference between the induration reactions promoted by the peptides antigens and the MST antigen were not significant. However, 72 h after the skin test, the results showed that the indurations promoted by PA3 were significantly different from the MST antigen. The difference is related to the average diameter of PA3 at 72 h (0.72 cm), whereas the MST antigen diameter was only 0.48 cm. This result confirms that PA3 demonstrated better performance when compared with the MST antigen because it can maintain a longer immunity response for a lengthier amount of time, thus facilitating the reading of the test in remote places or with difficult access to medical services.

Discussion

Several *Leishmania* sp. virulence factors have been identified, and the protein GP63 has been identified as the major surface

Table 1 Peptides sequence after chemical synthesis

	Sequence	Expected mass	Obtained Mass	aa[1] number
Peptide 1	G H β M P β T S V S β L A R P	1931.031 Da	1931.059 Da	15
Peptide 2	T β V P K E P β P L S G β R K	2021.124 Da	2021.203 Da	15
Peptide 3	S β P Q P N N F β L N S L G β	1985.048 Da	1985.121 Da	15

[1] Amino acid

glycoprotein of *Leishmania* sp. promastigotes forms. It represents more than 1% of the parasite's total protein (Yao et al. 2003; Link et al. 2017) and has been used in several experimental immunization studies with different *Leishmania* species and conferring effective protection on susceptible mice (Jaafari et al. 2007). For these reasons, this molecule could be used in diagnostic methods that evaluate a cellular immune response. Methods based on a cellular immunity response have been widely used in leishmaniases diagnosis. Among these methods, the MST can be highlighted for its sensitivity, specificity, low cost, easy application, and capacity to be performed in remote places. The current MST antigen was obtained by the extraction of proteins from promastigote forms of *Leishmania* sp. that diverge in the etiological agent depending on the region in the world. The lack of a standard antigen and other difficulties in the manufacturing process require new studies on the formulation of antigens with sensitivity and specificity to be applied on skin tests. To overcome these limitations, we proposed, in this work, the application of new biomolecules as antigens obtained by phage display for the skin test. For this, the biological response of the mimetic peptides as skin test antigens was evaluated in an animal model to verify whether or not these biomolecules could be used as antigen candidates in a hypersensitive reaction for a CL skin test.

The selected animal model was *C. porcellus* (guinea pig), which resulted in a suitable model to test the MST quality control process (Guedes et al. 2017) and could be used as an in vivo model for screening new biomolecules. Our observations are corroborated by other authors as this animal model is well established to study a DTH in different types of disease and is also used in the investigation of infectious diseases (Kim 2011; Komori et al. 2011; Malaghini et al. 2011; Kukharenko et al. 2015; Moradi et al. 2015). Secondly, the guinea pig has been established as an in vivo model for a CL skin test (Khabiri et al. 2006; Guedes et al. 2017).

The peptides that were evaluated in this study were fused to BSA, which is one of the most frequently used proteins to conjugate small molecules for vaccines or in immunoassay approaches (Lateef et al. 2007; Alban et al. 2013). Peptides are small molecules of nearly 2000 Da, which characterizes them as haptens. These molecules alone are unable to induce a specific immune response. However, they react with other molecules, and in this way, they can become immunogenic and able to promote humoral and cellular immune responses (Kindt et al. 2007).

The skin test response in *C. porcellus* immunized with *L. amazonensis* or *L. braziliensis* showed that the peptides antigens—individually (PA1, PA2, PA3) or in a mix (PAMix) promoted induration reactions of ≥ 0.5 cm after 48 h and remain positive for 72 h after the skin test. The DTH response promoted by peptides varied from 0.46 (PA1) to 0.61 cm (PA3) 48 h after the test in *C. porcellus* immunized with *L. amazonensis* (G1), and in *C. porcellus* immunized with *L. braziliensis* (G2), the indurations after 48 h varied from 0.55 (PA1) to 0.60 cm (PA3).

The statistical analysis of the skin test response of peptide antigens and the MST antigen in both groups (G1 and G2) showed that 48 h after a skin test, the difference between the induration reaction promoted by the peptides' antigens and the MST antigen was not significant. But, after 72 h, the indurations promoted by PA3 in the G1 group and PA2 and PAMix in the G2 group were significantly different from the MST antigen. These differences are due to the average diameter of the indurations which result from the injection of these peptide antigens. The average diameter of PA3 at 72 h was 0.72 cm, while it was 0.48 cm the for MST antigen (in G1). For PA2, the average diameter was 0.71 cm; for PAMix, it was 0.73 cm; and for the MST antigen, it was 0.45 cm (in G2). These results confirm that PA3, PA2, and PAMix are better than the MST antigen because they can maintain a longer immunity response for a lengthier amount of time, which is an advantage because it promotes a longer induration diameter and readout time of 72 h and permits a patient more time to go to a health service provider and receive a diagnosis.

This is the first study that reports the use of mimetic peptides as antigens in skin tests for CL. Other antigens, such as soluble *Leishmania* purified protein (Khabiri et al. 2005, 2006) and recombinant proteins, have been investigated (Teixeira et al. 2011). However, in these cases, the downstream process is more expensive and time consuming; further, in the case of recombinant proteins, they also require a step to remove lipopolysaccharides to avoid anaphylactic shock in patients. In comparison, mimetic peptides can be chemically produced and contain no contaminants.

Table 2 Average diameters (cm) of induration reactions promoted by new antigens (PA1, PA2, PA3, and PAMix) comparing to MST in *Cavia porcellus* immunized with *Leishmania amazonensis* 48–72 h after skin test

	Leishmania amazonensis			
	48 h		72 h	
Antigen	Mean (SD)[a] cm	95% CI[b]	Mean (SD)[a] cm	95% CI[b]
PA1[c]	0.47 (0.11)	0.36–0.58	0.46 (0.12)	0.34–0.58
PA2[d]	0.54 (0.07)	0.47–0.61	0.53 (0.08)	0.45–0.61
PA3[e]	0.61 (0.12)	0.49–0.73	0.72 (0.20)	0.52–0.92
PAMix[f]	0.54 (0.09)	0.45–0.63	0.58(0.11)	0.47–0.69
MST[g]	0.50 (0.00)	0.50–0.50	0.48 (0.04)	0.44–0.52

[a] Standard deviation

[b] 95% confidence interval

[c] Peptide antigen 1 average diameter of indurations and standard deviation at 48 and 72 h after the test

[d] Peptide antigen 2 average diameter of indurations and standard deviation at 48 and 72 h after the test

[e] Peptide antigen 3 average diameter of indurations and standard deviation at 48 and 72 h after the test

[f] Mix of peptide antigens (PA1 + PA2 + PA3) average diameter of indurations and standard deviation at 48 and 72 h after the test

[g] Montenegro skin test antigen. Average diameter of indurations and standard deviation at 48 and 72 h after the test

Table 3 Average diameters (cm) of induration reactions promoted by new antigens (PA1, PA2, PA3, and PAMix) in comparison to MST antigen in *Cavia porcellus* immunized with *Leishmania braziliensis* 48–72 h after skin test

	Leishmania braziliensis			
	48 h		72 h	
Antigen	Mean (SD)[a] cm	95% CI[b]	Mean (SD)[a] cm	95% CI[b]
PA1[c]	0.55 (0.13)	0.42–0.68	0.57 (0.10)	0.47–0.67
PA2[d]	0.56 (0.04)	0.52–0.60	0.71 (0.22)	0.49–0.93
PA3[e]	0.61 (0.07)	0.54–0.68	0.68 (0.07)	0.61–0.75
PAMix[f]	0.59 (0.13)	0.46–0.72	0.74 (0.15)	0.59–0.89
MST[g]	0.51 (0.02)	0.49–0.53	0.45 (0.05)	0.40–0.50

[a] Standard deviation

[b] 95% confidence interval

[c] Peptide antigen 1 average diameter of indurations and standard deviation at 48 and 72 h after the test

[d] Peptide antigen 2 average diameter of indurations and standard deviation at 48 and 72 h after the test

[e] Peptide antigen 3 average diameter of indurations and standard deviation at 48 and 72 h after the test

[f] Mix of peptide antigens (PA1 + PA2 + PA3) average diameter of indurations and standard deviation at 48 and 72 h after the test

[g] Montenegro skin test antigen. Average diameter of indurations and standard deviation at 48 and 72 h after the test

The peptide antigens used in this work are mimotopes that have homology with two *Leishmania* proteins: GP63 and LPG. These proteins are virulence factors of *Leishmania* sp. and modulate or interfere with the recognition of patterns by toll-like receptors (TLRs). This regulation induces the production of signals responsible for the activation of genes that are important for an immune response. In this way, the peptides are capable of stimulating the production of pro-inflammatory cytokines responsible for a cellular immunity response.

It is interesting to highlight that CL is one of the six endemic diseases in the world. It is considered a neglected disease that affects people all around the world and the majority of them are in low-income areas. It is a social and health problem, so it is necessary to invest in early diagnosis with methodologies that are highly sensitive, specific, inexpensive, easy to perform and transport, and are in a suitable treatment as a tool to control the disease. The skin test is one, of these methodologies, that is widely used as a complementary tool by a healthcare worker to investigate the disease in CL-

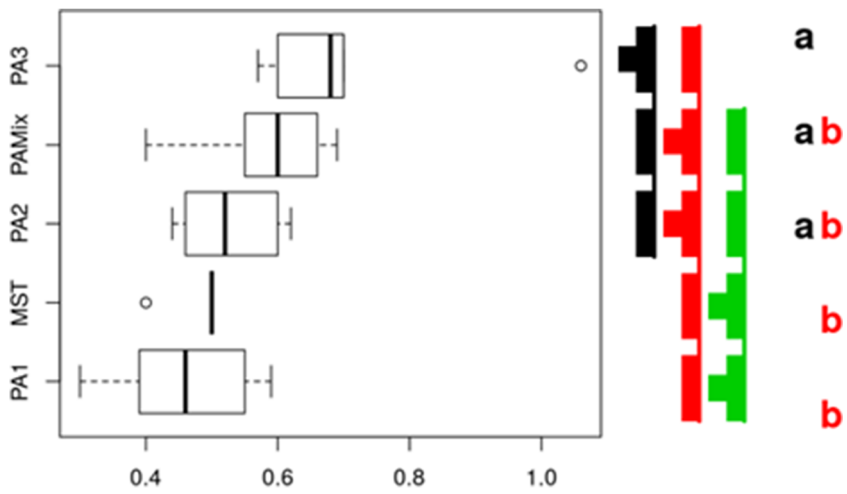

Fig. 2 Statistical analysis of induration reactions promoted by PA1, PA2, PA3, PAMix, and MST antigens 72 h after skin test in *Cavia porcellus* immunized with *Leishmania amazonensis*. PA1: peptide antigen 1; PA2: peptide antigen 2; PA3: peptide antigen 3; PAMix: mix of peptide antigen (PA1 + PA2 + PA3); MST: Montenegro skin test

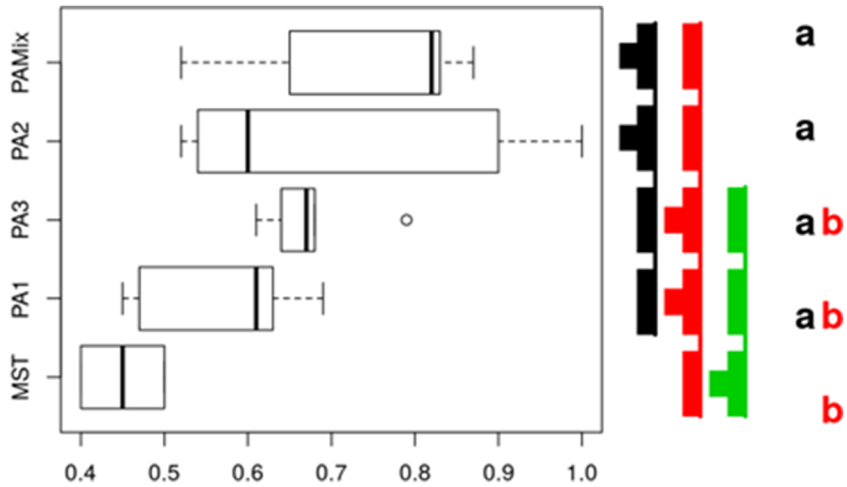

Fig. 3 Statistical analysis of induration reactions promoted by PA1, PA2, PA3, and PAMix compared with MST antigen 72 h after skin test in *Cavia porcellus* immunized with *Leishmania braziliensis*. PA1: peptide antigen 1; PA2: peptide antigen 2; PA3: peptide antigen 3; PAMix: mix of peptide antigen (PA1 + PA2 + PA3); MST: Montenegro skin test

endemic areas, being the first approach to verify whether or not the patient is reactive. To confirm the disease, it is necessary to perform more diagnostics, such as clinical, epidemiological, immunological, and molecular methodologies, in healthcare centers.

The peptides can recruit an immune response that is equal to or longer than that observed for the MST antigen and can maintain the induration reactions for a longer period of time. This characteristic is desired in CL-endemic areas, including impoverished regions in developing countries where access to hospitals and healthcare is limited, thus making it difficult for patients to return for final diagnoses. Therefore, the antigen for a skin test must be able to maintain a positive immune response for longer, thereby making it possible for patients to return to hospitals to receive final diagnoses.

Based on the results of this work, our research group is looking forward to developing new studies with these peptides to improve their application as antigens for a leishmaniases skin test diagnosis. Good perspectives are being discussed to evaluate their antigenic ability to be applied in a skin test with other animal models suitable for *Leishmania* infection and to test them with a larger number of animals per group. We expect that these further studies will lead us to investigate the potency of these peptides in human patients and their application as skin test antigens can possibly lead to a better diagnostic test that will lend itself to an early diagnosis of leishmaniases.

In summary, our results confirm that the PA3 antigen is better than the MST antigen because it can maintain a longer immunity response for longer, thus facilitating skin test readings in remote places or with difficult access to medical services. PA2 and PAMix demonstrated good results and were significantly different from the MST antigen when the animals were sensitized with *L. braziliensis*. The peptides evaluated here for the CL skin test are promising antigen candidates for producing a new skin sensitivity test that can contribute to its application in endemic areas of Brazil and worldwide.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was approved by the Research Ethics Committee of the Federal University of Parana (Process no. 101328/2015-69). This article does not contain any studies that were performed by any of the authors with human subjects.

Conflict of interest The authors declare that they have no competing interests.

References

Alban SM, de Moura JF, Minozzo JC, Mira MT, Soccol VT (2013) Identification of mimotopes of *Mycobacterium leprae* as potential diagnostic reagents. BMC Infect Dis 13. https://doi.org/10.1186/1471-2334-13-42

Alban SM, De Moura JF, Thomaz-Soccol V et al (2014) Phage display and synthetic peptides as promising biotechnological tools for the serological diagnosis of leprosy. PLoS One 9:e106222. https://doi.org/10.1371/journal.pone.0106222

Bazan J, Całkosiński I, Gamian A (2012) Phage display - a powerful technique for immunotherapy: 2. Vaccine delivery. Hum Vaccin Immunother 8:1829–1835

Brigido M, Maranhão A (2002) Bibliotecas apresentadas em fagos. Biotecnologia cienc desenvolv 26:44–51

Capelli-Peixoto J, Chávez-Olórtegui C, Chaves-Moreira D, Minozzo JC, Gabardo J, Teixeira KN, Thomaz-Soccol V, Alvarenga LM, de Moura J (2011) Evaluation of the protective potential of a *Taenia*

solium cysticercus mimotope on murine cysticercosis. Vaccine 29: 9473–9479. https://doi.org/10.1016/j.vaccine.2011.10.027

Charan J, Kantharia ND (2013) How to calculate sample size in animal studies? J Pharmacol Pharmacother 4:303–306. https://doi.org/10.4103/0976-500X.119726

Chen X, Scala G, Quinto I, Liu W, Chun TW, Shawn Justement J, Cohen OJ, vanCott TC, Iwanicki M, Lewis MG, Greenhouse J, Barry T, Venzon D, Fauci AS (2001) Protection of *Rhesus macaques* against disease progression from pathogenic SHIV-89.6PD by vaccination with phage-displayed HIV-1 epitopes. Nat Med 7:1225–1231. https://doi.org/10.1038/nm1101-1225

de Moura J, Alvarenga LM, Thomaz-Soccol V (2016) Biotechnological role of phage-displayed peptides for the diagnosis of neglected tropical diseases. In: Current developments in biotechnology and bioengineering: human and animal health applications. pp 161–180

de Vries HJC, Reedijk SH, Schallig HDFH (2015) Cutaneous leishmaniasis: recent developments in diagnosis and management. Am J Clin Dermatol 16:99–109. https://doi.org/10.1007/s40257-015-0114-z

Fogaça RL, Capelli-Peixoto J, Yamanaka IB, de Almeida RPM, Muzzi JCD, Borges M, Costa AJ, Chávez-Olortegui C, Thomaz-Soccol V, Alvarenga LM, de Moura J (2014) Phage-displayed peptides as capture antigens in an innovative assay for *Taenia saginata*-infected cattle. Appl Microbiol Biotechnol 98:8887–8894. https://doi.org/10.1007/s00253-014-5968-0

Guedes DC, Minozzo JC, Pasquali AKS, Faulds C, Soccol CR, Thomaz-Soccol V (2017) New strategy to improve quality control of Montenegro skin test at the production level. Rev Soc Bras Med Trop 50:788–794. https://doi.org/10.1590/0037-8682-0131-2017

Hancock DC, O'Reilly NJ (2005) Synthetic peptides as antigens for antibody production. In: Immunoch protocols. pp 13–25

Harlow D, Lane E (1988) A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Jaafari MR, Badiee A, Khamesipour A, Samiei A, Soroush D, Kheiri MT, Barkhordari F, McMaster WR, Mahboudi F (2007) The role of CpG ODN in enhancement of immune response and protection in BALB/c mice immunized with recombinant major surface glycoprotein of *Leishmania* (rgp63) encapsulated in cationic liposome. Vaccine 25: 6107–6117. https://doi.org/10.1016/j.vaccine.2007.05.009

Khabiri AR, Bagheri F, Alimohammadian MH, Assmar M, Nadaf SR (2005) Leishmanin skin test in guinea pig with a single purified protein of *Leishmania major*. Exp Parasitol 111:239–243. https://doi.org/10.1016/j.exppara.2005.08.011

Khabiri AR, Bagheri F, Assmar M (2006) *Leishmania major*: species specific delayed hypersensitivity reaction induced by exogenous secreted antigen in the guinea pig. Exp Parasitol 112:184–186. https://doi.org/10.1016/j.exppara.2005.11.005

Kim YJ (2011) Efficiency of recombinant bacille calmette-guérin in inducing humoral and cell mediated immunities against human immunodeficiency virus type 1 third variable domain in immunized mice. Yonsei Med J 52:173–180. https://doi.org/10.3349/ymj.2011.52.1.173

Kindt T, Goldsby R, Osborne B, Kuby J (2007) Kuby immunology. J Exp Med 206:2925–2935. https://doi.org/10.1017/CBO9781107415324.004

Komori T, Nakamura T, Matsunaga I, Morita D, Hattori Y, Kuwata H, Fujiwara N, Hiromatsu K, Harashima H, Sugita M (2011) A microbial glycolipid functions as a new class of target antigen for delayed-type hypersensitivity. J Biol Chem 286:16800–16806. https://doi.org/10.1074/jbc.M110.217224

Kukharenko AE, Babaev AA, Shchelchkova NA, Lapshin RD, Vedunova MV, Gravel IV, Mukhina IV, Lovtsova LV (2015) Estimation of general toxicity and immunological safety of a novel therapeutic vaccine against human papillomavirus-associated diseases. Sovrem Technol Med 7:92–96. https://doi.org/10.17691/stm2015.7.2.12

Lateef SS, Gupta S, Jayathilaka LP et al (2007) An improved protocol for coupling synthetic to carrier proteins for antibody production using DMF to solubilize peptides. J Biomol Tech 18:173–176

Link JS, Alban SM, Soccol CR, Pereira GVM, Thomaz Soccol V (2017) Synthetic peptides as potential antigens for cutaneous leishmaniosis diagnosis. J Immunol Res 2017:1–10. https://doi.org/10.1155/2017/5871043

Malaghini M, Thomaz-Soccol V, Probst CM, Krieger MA, Preti H, Kritski A, Soccol CR (2011) Recombinant antigen production for assays of intradermoreaction for diagnosis and surveillance of tuberculosis. J Biotechnol 156:56–58. https://doi.org/10.1016/j.jbiotec.2011.07.015

Merrifield R (1969) Solid-phase peptide synthesis. Adv Enzym 32:221–296

Moradi J, Mosavari N, Ebrahimi M, Arefpajohi R, Tebianian M (2015) Evaluation of *Mycobacterium tuberculosis* early secreted antigenic target 6 recombinant protein as a diagnostic marker in skin test. Osong Public Health Res Perspect 6:34–38. https://doi.org/10.1016/j.phrp.2014.12.002

Nagill R, Kaur S (2011) Vaccine candidates for leishmaniasis: a review. Int Immunopharmacol 11:1464–1488

R Development Core Team R (2011) R: a language and environment for statistical computing. R Found Stat Comput 1:409

Sadeghian G, Ziaei H, Bidabadi L, Nilforoushzadeh M (2013) Evaluation of leishmanin skin test reaction in different variants of cutaneous leishmaniasis. Indian J Dermatol 58:239. https://doi.org/10.4103/0019-5154.110838

Skraba CM, de Mello TFP, Pedroso RB et al (2015) Evaluation of the reference value for the Montenegro skin test. Rev Soc Bras Med Trop 48:437–444. https://doi.org/10.1590/0037-8682-0067-2015

Szargiki R, Alcântara de Castro E, Luz E et al (2009) Comparison of serological and parasitological methods for cutaneous leishmaniasis diagnosis in the state of Paraná, Brazil. BJID 12:47–52

Teixeira MCA, de Sá Oliveira GG, Santos POM et al (2011) An experimental protocol for the establishment of dogs with long-term cellular immune reactions to Leishmania antigens. Mem Inst Oswaldo Cruz 106:182–189. https://doi.org/10.1590/S0074-02762011000200011

Thomaz-Soccol V, Alban S, Seger J (2015) Peptídeos miméticos de *Leishmania* sp. Processo para sua obtenção e aplicações. Patent

Wang H, Gao Y, Gong Y, Chen X, Liu C, Zhou X, Blackall PJ, Zhang P, Yang H (2007) Identification and immunogenicity of an immunodominant mimotope of *Avibacterium paragallinarum* from a phage display peptide library. Vet Microbiol 119:231–239. https://doi.org/10.1016/j.vetmic.2006.08.034

WHO (1997) World Health Organization expert committee on biological standrdization - technical report series 889, pp 1–120

Wu HW, Hu XM, Wang Y, Kurtis JD, Zeng FJ, McGarvey ST, Wu GL, Zhang ZS, Hua ZC (2006) Protective immunity induced by phage displayed mitochondrial related peptides of *Schistosoma japonicu*m. Acta Trop 99:200–207. https://doi.org/10.1016/j.actatropica.2006.08.006

Yao C, Donelson JE, Wilson ME (2003) The major surface protease (MSP or GP63) of *Leishmania* sp. biosynthesis, regulation of expression, and function. Mol Biochem Parasitol 132:1–16

Zurlo J, Rudacille D, Goldberg AM (1996) The three Rs: the way forward. Environ Health Perspect 104:878–880