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Immunodetection of the “brown” spider (*Loxosceles intermedia*) dermonecrotoxin with an scFv-alkaline phosphatase fusion protein

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ABSTRACT

Bites by spiders from *Loxosceles* genus often lead to a wide variance in envenomation profile of patients and diagnosis is difficult due to the number of diseases that mimic loxoscelism. In such a context, it is of interest to consider the design of standardized recombinant colorimetric antibodies for diagnosis and specific detection of individual circulating toxins in biological fluids of envenomed patients. We have previously prepared a monoclonal murine IgG (LiMab7) that reacts with *Loxosceles intermedia* venom components of 32–35 kDa and neutralizes the dermonecrotic activity of the venom. Here, we re-engineered LiMab7 into a colorimetric bifunctional protein consisting in the corresponding single-chain antibody fragment (scFv) fused to alkaline phosphatase (AP) of *Escherichia coli*. The immune tracer was tested in two different types of immunoassays and it proved to be efficient in both. Thus, this recombinant fusion protein (scFv-LiMab7/AP) can be used for rapid and specific immunotitration of *L. intermedia* venom with a linear range of 39–20000 ng/mL and a detection limit of 39 ng/mL without any cross-reaction.
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1. Introduction

“Brown recluse”, “brown fiddler”, “fiddleback”, “violin” or “recluse” spider in North America or, simply, “brown” spider in South America, are some popular names used to refer to arachnids of the genus *Loxosceles* (Araneae, Sicariidae), although they can be found worldwide [1].

Even though the genus *Loxosceles* comprises more than 100 species and all of them are probably capable of producing medically significant bites, the major spider responsible for envenomation in United States is *Loxosceles reclusa*, while *Loxosceles intermedia*, *Loxosceles gaucho* and *Loxosceles laeta* are considered to be the most important from a medical point of view in South America [2–5]. In Brazil, the brown spider envenoming is considered as a serious public health threat due to the number of cases recorded annually. In 2012, 25,329 spider bites were recorded. It is estimated that approximately 50% of these bites are caused by spiders belonging to the genus *Loxosceles* [6].

Typically, clinical picture is a necrotizing-hemolytic syndrome starting with local oedema and ischemia at the bite site followed by frank necrosis (eschar) within days and occasionally extended necrotic areas over weeks. A small subset of patients can even have a more severe systemic response causing hemolysis and rhabdomyolysis with subsequent anemia, renal failure and hematuria which may be more pronounced in children [7,8].

Loxoscelism diagnostic is tedious because bites often go unnoticed. The early clinical signs are not specific and by the time a necrotic lesion develops it may be too late for interventions and full rescue. Therefore, laboratory immunoassays should be developed beside to conventional clinical tests in order to facilitate the specific detection of the toxins into biological fluids. Such immunoassays, more likely ELISA, are usually developed using conventional antibodies (polyclonal or monoclonal) with several drawbacks: (i) maintaining a reproducible source of polyclonal antibodies is

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difficult, (ii) monoclonal antibodies production from hybridoma is labour intensive, requires expensive cell culture facilities and finally, leads to immunoprobes of which specificity and affinity are fixed and can't be altered. In addition, current methods for the synthesis of antibody conjugates often lead to heterogeneous molecules with altered functional properties.

The characterisation of murine monoclonal antibody LiMab7 which is directed against toxic components of *L. intermedia* venom was previously reported [9]. Now, this antibody can be considered as a starting material to design recombinant immunoconjugates with diagnostic and therapeutic potential. Here, we produced and characterized a recombinant colorimetric immunotracer made of the single chain antibody fragment (scFv LiMab7) fused with the alkaline phosphatase (AP) of *Escherichia coli*. This technology simplifies the production of immunotracers and has advantages over conventional chemical conjugation of whole IgG molecules [10].

Here, simple ELISAs that allow to detect *L. intermedia* venom component in biological fluids using the scFv-LiMab7/AP recombinant conjugate were set up. Such recombinant immunotracer made by the fusion of an antibody fragment with an enzyme could become a valuable tool to develop robust immunoassays that should facilitate a rapid and reliable diagnostic.

2. Materials and methods

2.1. Venoms, toxin and antibodies

Venoms were obtained from *L. laeta*, *L. gaucho*, and *L. intermedia* spiders. The spiders, which were taxonomically identified and captured in various areas of Curitiba city (PR, Brazil), were provided by the Center for Production and Research of Immunobiological Products (CPPI, Piraquara, PR, Brazil). Venoms were collected by electrical stimulation applied to the cephalothorax of the spiders. Subsequently, venoms were vacuum dried, filtered, and stored at −20 °C. The recombinant dermonecrotic protein (rLiD1) was produced as previously described [11]. Total protein concentration was evaluated by a bicinchoninic acid assay (Pierce). Hybridoma LimAb7 secretes a well-characterized IgG specific for the venom of *L. intermedia* [9]. Specific horse polyclonal antibodies to *L. intermedia* were purified in a single step by immunoaffinity chromatography using *L. intermedia* venom immobilized on CNBr-Sepharose column (GE-Healthcare) as previously reported [12].

2.2. Construction of single-chain antibody fragment genes

Total RNA was isolated from freshly subcloned hybridoma LimAb7 cells secreting an IgG_{1k}. cDNAs encoding the antibody variable domains (IGHV and IGKV) were cloned after RT-PCR using degenerate primers IGH-For (GAC AGT GGA TAR ACM GAT GG) and IGH-Rev (GAG GTS MAR CTG CAG SAG TCW GG) for LimAb7 IGHV amplification, and V- KAPPA-Rev (GAT ATT GTT CTC ACC CAG TCT) and V-KAPPA-For (GGA TAC AGT TGG TGC AGC ATC) for LimAb7 IGKV amplification. Then, a synthetic gene encoding LimAb7 IGHV fused to LimAb7 KAPPA via a $(Gly_4Ser)_3$ peptide linker was designed with codon sequence optimized for prokaryotic expression and terminal restriction sites chosen for in frame cloning into various expression vector (pLip6/GN, pET22b(+) and others). A step by step protocol has been reported previously [13].

2.3. Gene synthesis and construction of expression vector

cDNA encoding scFv-LimAb7 (GenBank accession number KT381972) was cloned into pLip6/GN prokaryotic expression vector kindly provided by Dr Frédéric Ducancel (CEA,/Saclay, Gif-sur-Yvette, France). This vector has been previously reported for expression of scFv in fusion with *E. coli* alkaline phosphatase [14]. Following double digestion with *Sfi*I and *Not* I, the gene was introduced into the plasmid and the ligation product was transformed into *E. coli* strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [Fí *proAB lacIqZ*Δ*M15* Tn*10* (Tetr)] Agilent (cat. no. 200150). All basic molecular biology procedures were carried out as in Ref. [15].

The transformed bacteria were spread on Luria–Bertani agar plates containing ampicillin (0.05 g/L), IPTG (0.5 mM), and 5-bromo-4-chloro-3-indolyl phosphate (80 μg/mL). The phosphate in the medium inhibited the expression of bacterial endogeneous AP gene, and IPTG induced the lac promoter, allowing the expression of AP fusion proteins that hydrolyzed 5-bromo-4-chloro-3-indolyl phosphate to produce blue colonies. A positive ampicillin resistant blue colony was selected, and cultured in 2× yeast extract and tryptone medium (2XTY) with ampicillin (0.05 g/L) at 37 °C until the A_{600nm} reached approximately 0.6–1.0. The promoter was then induced with IPTG (0.8 mM) overnight. Bacteria from cultures were centrifuged, and the supernatant was separated from the pellet. The pellet was resuspended in saccharose solution (20% saccharose, 0.3 mol/L Tris–HCl, pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid), and periplasmic proteins were extracted by cold osmotic shock. The suspension was centrifuged for 10 min at 10,000 *g* at 4 °C, and the supernatant containing soluble fusion protein was collected. The fusion protein was detected by both SDS-PAGE and Western blotting with polyclonal rabbit antibodies against AP (ref 200–4134, Tebu, F). Samples were extensively dialyzed against Phosphate Buffer saline (PBS), pH 7.4.

2.4. Identification of the scFv-LimAb7/AP fusion protein and specificity characterization

2.4.1. Expression and identification of the scFv-LimAb7 fusion protein

Expression of the fusion protein was analysed after 10% SDS-PAGE electrophoresis followed by Coomassie Brilliant Blue staining or overnight passive transfer onto 0.45 mm nitrocellulose membrane. After blocking the membrane with 5% (w/v) non-fat dry milk in PBS, transferred proteins having AP activity were detected by incubation with AP substrate, BCIP/NBT liquid substrate (Sigma-Aldrich).

The AP activity of periplasmic extracts was measured in a Beckman Coulter DxC 800 automat using standard procedures.

2.4.2. Assay specificity

L. laeta, *L. gaucho*, and *L. intermedia* venoms and rLiD1 were subjected to SDS-PAGE on a 12.5% acrylamide gel. Gels were run at 120 V for 1 h. After electrophoresis, gels were stained with Coomassie Brilliant blue for identification of protein bands or transferred onto a 0.45 mm nitrocellulose membrane. Non-specific binding sites were blocked for 1 h in PBS containing 5% (w/v) non-fat dry milk and 0.3% (v/v) Tween 20. Then, the membrane was incubated with periplasmic extracts containing the scFv-AP diluted 1:2 in PBS-T (PBS, containing 0.05% (v/v) Tween 20) for 1 h at room temperature. AP activity was detected by incubation with AP substrate, BCIP/NBT liquid substrate (Sigma-Aldrich).

As a control, purified IgG LiMab7 was used in place of periplasmic extracts and immunocomplexes were revealed as previously reported [9].

2.5. Sequence analysis and data banks search

To easily compare V-REGION sequences of immunoglobulins we used the ImMunoGenetTics (IMGT) unique numbering and standards that have been approved by the World Health Organization (WHO)/International Union of Immunological Societies (IUIS) Nomenclature Subcommittee for IG and TR. We used the Web

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