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A strategy for identifying serodiagnostically relevant antigens of *Leishmania* or other pathogens in genetic libraries

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Abstract

Different individuals, when infected with the same parasite, rarely produce antibodies against the same set of antigens. Indeed, unless a particular antigen happens to be recognized by antibodies in all individuals, the use of a single antigen in the serodiagnosis of parasitic diseases leads, invariably, to false-negative results. A straightforward method for pin-pointing, in genetic libraries, the precise antigens that would increase serodiagnostic assay sensitivities is presented. The method is based on the utilization of sera that produced false-negative results against previously available antigens. Employing this false-negative serum-selection methodology for the identification of new *Leishmania infantum* recombinant antigens (rAgs), the sensitivity of a dipstick assay for anti-*Leishmania* antibodies in a panel of sera from patients with visceral leishmaniasis was increased from 83.3% to 98.1%, without affecting its specificity, by the inclusion of a fifth and a sixth *L. infantum* rAg.

Keywords: Recombinant antigen; cDNA library; Serodiagnosis; Immunoassay sensitivity; *Leishmania infantum*; Visceral leishmaniasis

1. Introduction

Obtaining several milligrams of a pure protein from a complex microorganism was not an easy task a few years ago. The recombinant DNA technology, however, is changing this situation: many homogeneously pure microbial antigens can now be obtained in large amounts with relative ease. Serodiagnostic assays of several infectious diseases, therefore, increasingly employ recombinant [1–4] rather than native antigens. A major obstacle, however, limits the use of a single antigen in the serodiagnosis: the specificities of the immune responses against complex microorganisms vary in different individuals, and, to the authors' best knowledge, no single antigen is recognized by antibodies in all of them. For instance, even when relatively few sera from *Leishmania infantum*- ($n = 9$) or *Trypanosoma cruzi*-infected ($n = 8$) human beings, or from rabbits immunized with a mixture of keyhole limpet hemocyanin and *Mycobacterium tuberculosis* antigens in mineral oil-saline emulsion ($n = 7$), were analysed by Western blot against *L. infantum*, *T. cruzi* or cross-reactive *Trypanosoma brucei* lysates, respectively, no single antigenic band was

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clearly recognized by all tested sera (Fig. 1; unpublished data). The solution for this problem, however, may be the employment of more than one antigen in a serodiagnostic assay: this would maximize the chances of detecting antibodies in all positive sera.

To improve a serodiagnostic recombinant antigen (rAg) panel, it would be desirable to pin-point, in genetic libraries, precisely the phages encoding the rAgs that might increase the assay sensitivity, ignoring those phages encoding already available antigens. This may be a difficult task—the best represented (not necessarily the most immunogenic, i.e., serodiagnosis-relevant) antigens in a library, after been easily picked up, leave behind a high "background" of undesirable phages encoding them. This would obstruct the identification of the relatively small number of phages encoding the desired new antigens. A direct way for obtaining these relatively rare rAgs is to use, as a screening tool, exactly the sera previously producing false-negative results: these sera do not, by definition, recognize the already available antigens. This false-negative serum-selection strategy was employed as described below, in two quick runs, to identify two different *L. infantum* rAgs from a cDNA library. These rAgs, due to the very process through which they were obtained, are recognized by previously false-negative sera, and are therefore promising candidates to increase the sensitivity of a serodiagnostic assay for zoonotic visceral leishmaniasis (VL), a Latin American/Southern European disease caused by the *L. infantum* protozoan [5,6].

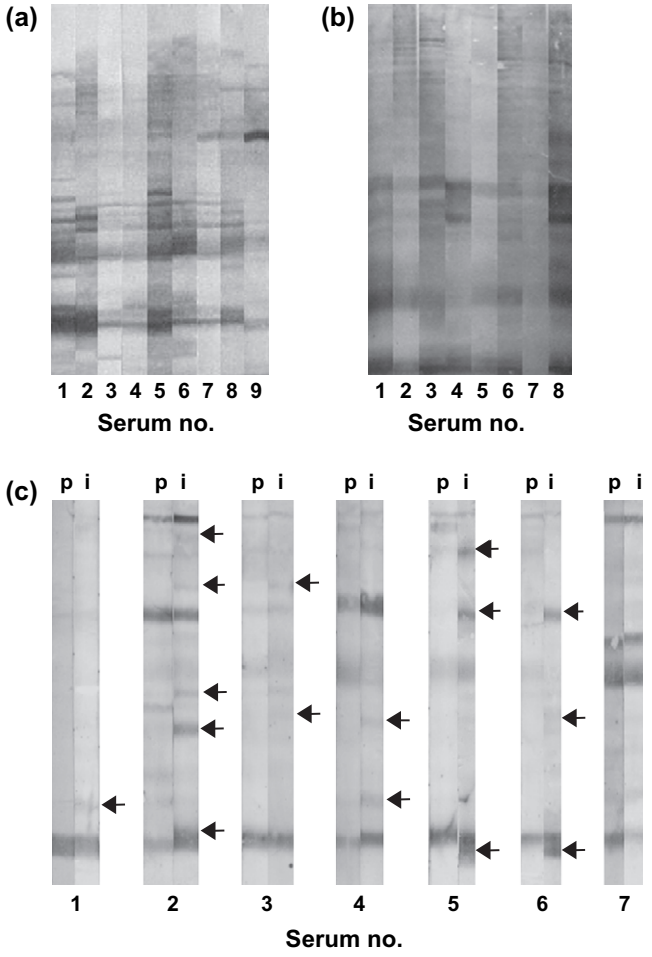

Fig. 1. Variability of immune responses in parasite-infected human beings or in animals immunized with complex antigenic mixtures. Sera from human beings naturally infected by *Leishmania chagasi* (a) or by *Trypanosoma cruzi* (b), and from New Zealand rabbits before and after immunization with a mixture of *Mycobacterium tuberculosis* and keyhole limpet hemocyanin (c), were used in Western blot assays against *L. chagasi*, *T. cruzi* and *Trypanosoma brucei* lysates, respectively. Each column corresponds to the reaction pattern of an individual serum. Arrows indicate antigens that were not recognized by antibodies in pre-immune serum. Note (i) how, although some antigens reacted strongly with antibodies from most human sera, none reacted with all sera (a and b), and (ii) the great variability of antibody specificities among individual rabbits, both in terms of "natural" (pre-immune) antibodies and of cross-reactive, post-immunization antibodies (c). p = pre-immune serum; i = immune serum.

2. Materials and methods

2.1. Sera

Serum samples from 54 VL patients, inhabiting an endemic area of the state of Rio Grande do Norte, in the dry, poor northeastern region of Brazil, were used. All patients had diagnoses confirmed by demonstration of the parasite in bone-marrow aspirates. Control sera were from 22 informed healthy volunteers without history of leishmaniases. All sera were prepared from blood collected for immunodiagnostic purposes and for the development of serodiagnostic assays, in accordance with institutional ethical guidelines (which include patients' informed consent).

2.2. Recombinant antigens and antigen-coating of nitrocellulose paper

Nitrocellulose paper was individually coated, as described below, with (i) *Leishmania* rAgs in *Escherichia coli* lysates; (ii) negative control *E. coli* lysate; and (iii) positive control *Leishmania* lysate.

L. infantum amastigotes were obtained from the spleens of hamsters infected with 10^8 metacyclic promastigotes and purified by centrifugation on a Percoll solution gradient [7]. The RNA isolated from purified amastigotes was used to construct a cDNA library in lambda ZAP bacteriophage (Stratagene, La Jolla, CA, USA) in accordance with manufacturer's instructions, in one of our laboratories. The library was screened with antibodies from pools of sera from (a) three dogs or (b) five human beings, all from VL endemic areas and with *Leishmania* amastigotes isolated from spleen or bone-marrow aspirates. Canine blood was collected by a veterinarian, in accordance with institutional ethical guidelines. Bacteriophages were isolated from the antibody-reacting plaques and incubated with *E. coli* agar cultures in Petri dishes, in the presence of isopropyl-β-D-thiogalactoside (IPTG), for the expression of the encoded recombinant proteins. The amount of added bacteriophages was that previously determined, by titration, to cause confluent lytic plaques on the bacterium layer