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Characterization of BIP protein of *G. lamblia* as a potential immunogen in a mouse infection model

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ABSTRACT

Giardia lamblia is a protozoan parasite that causes one of the most common gastrointestinal diseases worldwide. To eliminate the parasite from the host intestine, it is necessary the activation of B-cell and T-cell dependent mechanisms. The knowledge about *Giardia* antigens that can stimulate the host immune response is limited. Recently, it has been described the Binding Immunoglobulin Protein (BIP) of *G. lamblia* (71 kDa) as a potential immunogen. Additionally, our group has identified a highly immunogenic antigen (5G8 protein) of *G. lamblia* with a relative molecular mass of approximately 70 kDa. There is some evidence suggesting that the 5G8 protein may activate both humoral and cellular immune responses. Based on these observations and preliminary mass spectrometry analyses, we hypothesized that the antigen 5G8 could be the BIP protein. In the present study, we characterize immunochemically the BIP protein of *Giardia*. Flow cytometric assays and western blotting were used to determine the expression profile of BIP and 5G8 antigens in *Giardia* trophozoites. The differences in expression profile indicated that BIP and 5G8 are not the same molecule. ELISA and Western blotting assays revealed that BIP protein was recognized by antibodies produced during *G. lamblia* infection in C3H/HeN mice. MTT assays did not reveal the activation of cellular immune response induced by BIP protein *in vitro*. In addition, we identified the potential B-cell and T-cell epitopes of *G. lamblia* BIP protein. This molecule is a conserved protein among *Giardia* strains and other pathogens. The complete immunological characterization of this antigen will contribute to a better understanding of the host–parasite interactions in *Giardia* infection.

1. Introduction

Giardia lamblia is a binucleated flagellated protozoan that lives and reproduces in the small intestine of some mammals including humans (Adam, 2001; Luján et al., 2011). This parasite is the etiological agent of giardiasis, one of the most common gastrointestinal diseases worldwide (Adam, 2001). *Giardia* infection becomes a self-limited illness in over 85% of affected individuals, indicating the existence of effective host defense mechanisms against the parasite. Interestingly, cases of chronic giardiasis have been documented even in immunocompetent individuals (Eckmann, 2003).

It has been reported the importance of both T cells and B cells in anti-*Giardia* immunity. However, little is known about which *Giardia* antigens stimulate an effective immune response (Lopez-Romero et al., 2015). Additionally, most of the studies about *Giardia* immunogenic proteins, have been mainly focused on characterizing the host humoral response. We have previously described a group of protein bands from *Giardia* lysates (SDS-PAGE) that were recognized by secretory and systemic antibodies, using a model of *G. lamblia* infection in C3H/HeJ mice (Velazquez et al., 2005). During primary infection proteins of 63, 71 and 86 kDa were recognized, whereas, in secondary infection, additional 48, 55, 106 and 159 kDa proteins were detected. Furthermore, to evaluate T cell activation by *G. lamblia* proteins, we generated the first described *Giardia*-specific T cell hybridomas. Interestingly, proteins of 90–110 kDa, 65–77 kDa, and 40–64 kDa could stimulate several of those hybridomas (Astiazaran-Garcia et al., 2009), implying that proteins of ~70 kDa can stimulate a cellular immune response. Additional studies indicated that a 70 kDa protein (5G8) can stimulate host defenses to produce antibodies, which can induce *in vitro* agglutination of *Giardia* trophozoites (Quintero et al., 2013). Prelimin-

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ary mass spectrometry analysis (ESI-MS/MS) of the 70 kDa band (SDS-PAGE) identified the presence of the Binding Immunoglobulin Protein [BIP, a heat shock protein of 70 kDa (HSP 70)] among other proteins. It has been reported that *G. lamblia* BIP protein is potentially recognized by IgG antibodies from *G. lamblia*-infected BALB/C mice, and it also induced the activation of DCs and antigen presentation to naive T cells (Lee et al., 2014).

HSPs are chaperone proteins that are expressed constitutively in all cells, they contribute to cell surveillance under intrinsic or extrinsic stress conditions (Beckmann et al., 1992). The HSP70s have been reported in other organisms as being able to stimulate the immune response (Bolhassani and Rafati, 2008; Wang et al., 2015). In fact, HSP70s have been proposed as a candidate for vaccines or adjuvants against diseases and infections different from *Giardia* (Kaur et al., 2016; Dhakal et al., 2013; Segal et al., 2006; Assadian et al., 2014; Batra et al., 2014; Tamura et al., 2011; Tsukamoto et al., 2016; Li et al., 2016). The current knowledge regarding *Giardia* HSP70s is limited, members of this family have been used as molecular markers of organelles, life cycle stages (encystation) and giardiasis diagnosis (Stefanic et al., 2006; Lee et al., 2009; Soltys et al., 1996; Lujan et al., 1996; Kim et al., 2009). BIP protein, also known as GRP-78, is a HSP70 chaperone that was identified in *Giardia* as a resident protein of the endoplasmic reticulum, and it has been shown to be associated with encystation specific vesicles (ESV) (Stefanic et al., 2006; Soltys et al., 1996; Lujan et al., 1996). However, in human cells, specifically in tumor cells, BIP protein can also be found on the cell surface (Zhang et al., 2010). Although there is evidence that BIP protein has an immunogenic potential, it is still unknown whether this antigen may induce the development of effective immune mechanisms against *Giardia* that could lead to a protection to future *Giardia* challenges. The identification of immunoreactive proteins that can effectively stimulate B- and T-cell responses will be crucial for the development of new anti-*Giardia* drugs and vaccines. The aim of the present study was to determine whether BIP and 5G8 are the same antigen of *Giardia*, and to characterize the host humoral and cellular immune response against the BIP protein of *G. lamblia*.

2. Materials and methods

2.1. Mouse strain

C3H/HeN female mice of six to eight weeks old were used in the infection assays. Mice were bred at the University of Sonora bioterium with light/dark cycles of 12 h at 25 °C and sterile water and food *ad libitum*. Mice were housed and managed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Mexican Official Regulations (NOM-033; NOM-063).

2.2. G. lamblia trophozoite culture

G. lamblia trophozoites [clone GS/M-83-H7 (ATCC 50581)] were obtained from the American Type Culture Collection. *G. lamblia* strain GS/M-83-H7-5G8 (+), previously obtained by immunoabsorption assays (50-90% of the trophozoites express 5G8 antigen on the surface) (Quintero et al., 2013, 2017). Axenic *G. lamblia* cultures were maintained in the TYI-S-33 medium, supplemented with 10% newborn calf serum (NBCS) and antibiotics at 37 °C (Ceftriaxone 100 μg/mL).

2.3. G. lamblia protein lysates

G. lamblia soluble proteins were obtained using a modified method from the described by Gottstein (Gottstein et al., 1990). *G. lamblia* trophozoite cultures were harvested during log-phase, and incubated on ice-cold water during 15 min to detach cells. The harvested trophozoites were washed three times with sterile phosphate buffered saline (PBS). Afterwards, the trophozoites were lysed by three cycles of frozen (−80 °C) and thawed [room temperature (RT)] in the presence of a protease inhibitor cocktail [23 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, 0.3 mM pepstatin A, 0.3 mM E-64, 2 mM bestatin and 100 mM sodium ethylenediamine tetraacetic acid (Sigma, St. Louis, MO, USA)]. The *G. lamblia* lysate was sonicated three times during 60 sec and 10% amplitude (Brandon Sonifier S-250D, Shelton, CT, USA). Cell debris was removed by centrifugation (14,000 × *g* for 30 min). Protein concentration values were determined by the Bradford method (Bio-Rad, Hercules, CA, USA).

2.4. Expression plasmid design

The amino acid sequence of BIP protein (EES99538.1) from *G. lamblia* GS/M-83-H7 (ATCC 50581) was obtained from *Giardia* database (http://giardiadb.org/giardiadb/). To ensure the recombinant expression, N-terminal signal peptide and transmembranal residues were removed (47 residues starting from the amino end), considering that signal sequence is not present in the mature protein and could affect the expression efficiency in the recombinant system (Soltys et al., 1996). Additionally, we included a sequence 6xHIS (at the amino end), and a thrombin sensible sequence in order to ease future purifications. The modified sequence was cloned by DNA 2.0 (Newark, CA, USA) in the expression plasmid pJexpress404.

2.5. Recombinant protein expression and purification

We transformed the competent bacteria *E. coli* Rosetta gami with 10 ng of pJexpress404-BIP vector, and it was cultured in LB (Luria Bertani) agar plates. After 16 h culture, positive colonies were selected by their ampicillin resistance (100 μg/mL). To obtain soluble recombinant BIP (rBIP) protein, we optimized the over-expression conditions. Over-expression was carried by culturing 1 L of transformed *E. coli* Rosetta gami bacteria in Terrific Broth (TB) medium. Once the culture reached $D.O._{600\ nm}$ = 0.6–0.8 it was induced with 0.5 mM IPTG during 4 h at 37 °C. Cells were pelleted by centrifugation at 3600 × *g*, 30 min, 4 °C and washed twice with ice-cold PBS. After this step cells were always on ice. Then, the pellet was resuspended in column binding buffer (4 μL of buffer per 1 μL of pellet). Bacteria were lysed enzymatically adding lysozyme to the cell suspension at a final concentration of 2 mg/mL, and then lysed mechanically by sonication [6 cycles of 10 seconds pulses each and 20% amplitude (Brandon Sonifier S-250D, Shelton, CT, USA)]. Soluble proteins were separated by centrifugation at 10 000 × *g*, 30 min, 4 °C. The supernatant was collected and clarified by filtration through with 0.45 μm membranes. rBIP protein was purified by IMAC chromatography [pre-packed GE HisTrap column (1 ml)], the clarified sample was passed through the column with a peristaltic low pressure pump (Bio-Rad, Hercules, CA, USA) at a flow rate 0.2 mL/min. Protein was eluted using six buffer solutions at different imidazole concentrations (5, 20, 50, 100, 200, 500 mM). rBIP purified protein was obtained at buffer solution with the highest imidazole concentration with a purity of at least 80%.

2.6. Mouse infection

Five million trophozoites were resuspended in 200 μL of sterile PBS and administered by a sterile animal feeding needle for peroral inoculation. Primary infection occurred at day 0, while the second challenge took place at day 42. The infected and reinfected mice were bleed from the tail vein weekly during 6 weeks post-infection (PI), and post-reinfection (PRI). Serum was recovered and stored at −80 °C.

2.7. Generation of polyclonal antibodies specific to rBIP protein and Giardia antigens

To generate anti-*Giardia* polyclonal antibodies, six to fourteen weeks old C3H/HeN mice were inoculated intraperitoneally with *G. lamblia* lysate (200 μg) emulsified with an equal volume of Freund's

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