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Research Note

Gnathostoma binucleatum antigens induce peripheral blood lymphocytic cells proliferation

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Summary

Gnathostomiasis is an emerging infectious disease, in which the agent *Gnathostoma binucleatum* is the only species known to infect humans in Mexico. Although the humoral immune response to *G. binucleatum* has been analyzed previously, here we evaluated the lymphoproliferative response of peripheral blood lymphocytic cells from patients clinically diagnosed with gnathostomiasis against a crude extract of *G. binucleatum* using the MTT method, and compared the response to that of healthy subjects. No differences in the response to concanavalin A were found between the groups; however, the response to the crude extract was statistically higher in patients compared to healthy controls (1.188 ± 0.135, and 1.000 ± 0.045, p = 0.0004). Patients at > 6 months after diagnosis showed a higher lymphoproliferative response than the group of healthy subjects, and those at < 6 months after diagnosis (p = 0.0001, and 0.0164 respectively). These data suggest that the parasite induces non-protective immunological memory.

Keywords: Gnathostomiasis; Cellular immune response; *G. binucleatum*; non-protective immunity

Introduction

Gnathostomiasis is an emerging infectious disease in Mexico. More than 1500 human cases distributed in seven states along the Atlantic and Pacific slopes have been registered (Léon-Règagnon *et al*., 2002). Epidemiological studies have revealed that more than 2000 cases of this disease were reported in Mexico between 1999 and 2003, and that of those more than 500 were reported in the state of Nayarit (Lamothe-Argumedo, 2003). Although 18 species of *Gnathostoma* have been recognized worldwide (Bertoni-Ruiz *et al*., 2005), *G. binucleatum* is the only species known to infect humans in Mexico to date, though it is possible that other species might infect humans as well (Hernandez-Gomez *et al*., 2010).

In previous work, we analyzed the humoral immune response to *G. binucleatum* (Zambrano-Zaragoza *et al*., 2012); thus, the aim of this study was to analyze the lymphoproliferative response of peripheral blood lymphocytic cells (PBLC) from patients clinically diagnosed with Gnathostomiasis to *G. binucleatum* antigens.

Material and methods

Fourteen patients with clinical diagnoses of Gnathostomiasis according to clinical criteria (Zambrano-Zaragoza *et al*., 2012) examined at the General Hospital in Tepic, Nayarit, Mexico, were included in this study. They were divided into groups as follows: 1) Group A, 5 patients at < 6 months after diagnosis; and, 2) Group B, 9 patients at > 6 months after diagnosis. Fourteen healthy subjects with no history of cutaneous or subcutaneous migratory swelling, no previous symptoms compatible with migratory swelling, and no history of eating raw or uncooked fish were also included as control group. All participants were informed as to the nature of the study, and written consent was obtained according to the Helsinki Declaration. The study was approved by the local ethics committee. Blood samples were taken by venipuncture, and PBLC were obtained by Ficoll-Hypaque gradient.

To obtain the crude extract, approximately 500 ADVL3 were isolated from fish (*Cathorops fuerthii*), as described previously (5). They were then suspended in an extraction solution (1 % Triton X-100, 0.05 mM TLCK, 1 mM PMSF, 5 μg/mL Pepstatin A, and 41.54 mM EDTA), and lysated using a tissue homogenizer (Pro-Scientific PRO250) at 18,000 rpm for 5 min, followed by sonication

at 20 kHz for 6 min in an ice bath (Ultrasonic Processor GE-130). After centrifugation at 14000 rpm (Eppendorf 5810R) for 15 min at 4 ºC, the supernatant was recovered, and protein quantification was carried out using the bicinchoninic acid method (Pierce, Rockford, USA). The crude extract was then stored in aliquots at -20 ºC.

The lymphoproliferation of PBLC was determined by lymphoproliferative assay using MTT dye [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide], according to the method described by Mosmann (1983). Briefly, PBLC from patients and controls were isolated from heparinized peripheral blood by density gradient centrifugation using a Ficoll-Hypaque (GE Healthcare, Buckinghamshire England). A total of $2x10^5$ viable cells re-suspended in 200 µL of AIM-V medium (Gibco, Auckland, USA) were placed in each well of 96 U-well polystyrene plates (Nunc, Roskilde, Denmark). Cells were stimulated with 0.2 µg of a crude extract of *G. binucleatum*. Additionally, PBLC incubated in AIM-V medium alone, and PBLC stimulated with 1 µg of concanavalin A (ConA, Sigma chem. Co. St. Louis MO USA) were included as negative and positive controls, respectively. Plates were incubated at 37 ºC in 5 % CO_2 for 5 days. Afterwards, 100 µL supernatants were removed and 10 µL of 5 mg/mL MTT (Calbiochem, Darmstadt Germany) solution were added, followed by incubation for 4 hours at 37 ºC. Next, 100 µL of lysis solution (10 % SDS in 0.01N HCl) were added and incubated for an additional 18 hours to dissolve the formazan crystals. Optical density (OD) was then measured at a test wavelength of 595 nm in a microplate reader at 595 nm (BIO-RAD Model 680 Microplate Reader). Results were expressed as stimulation indexes (SI) calculated by dividing the mean of OD_{595nm} of stimulated PBLC by the mean of OD_{595nm} of the negative control. The mean absorbance obtained in each condition was compared between patients and healthy subjects using the Mann-Whitney test with a 95 % confidence interval and Minitab® software, release 14.13.

Results and discussion

As shown in Figure 1, we found that the PBLC from patients showed a lymphoproliferative response to Con A (mean 2.447 ± 0.895) similar to that observed in healthy subjects (mean 2.098 ± 0.815). No differences in the SI of PBLC were observed between groups (p = 0.2413), and the response was similar in males and females in the control and patients groups (p = 0.7768, and 0.5613 respectively). These results indicate that the PBLC from all subjects included in this study were functional and had the ability to proliferate.

Although all subjects analyzed in this study showed a lymphoproliferative response to *G. binucleatum* antigens, the patients clinically diagnosed with gnathostomiasis had higher SI (mean 1.188 ± 0.135) than healthy subjects (mean 1.000 ± 0.045, p = 0.0004). These data, together with those reported about the humoral immune response (Zambrano-Zaragoza *et al*., 2012), strongly suggest that those patients have clones of T and B lymphocytes that recognized antigens from *G. binucleatum*, unlike control subjects.

When data from the patients were analyzed according to group (Fig. 2), the study found that the group A patients had a lower lymphoproliferative response than group B

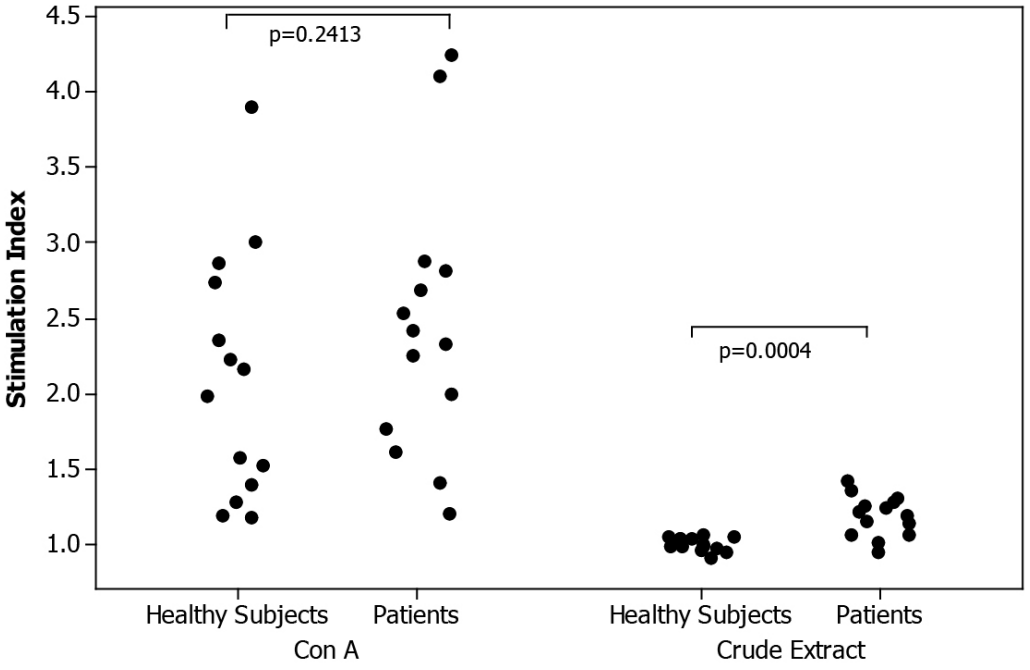

Fig. 1. Lymphoproliferative response to Con A and a crude extract from *Gnathostoma binucleatum* in patients clinically diagnosed with gnathostomiasis and healthy subjects (control), determined by MTT assay. The graph shows the individual IE values for each group. Statistical analysis was done using the Mann-Whitney U test.

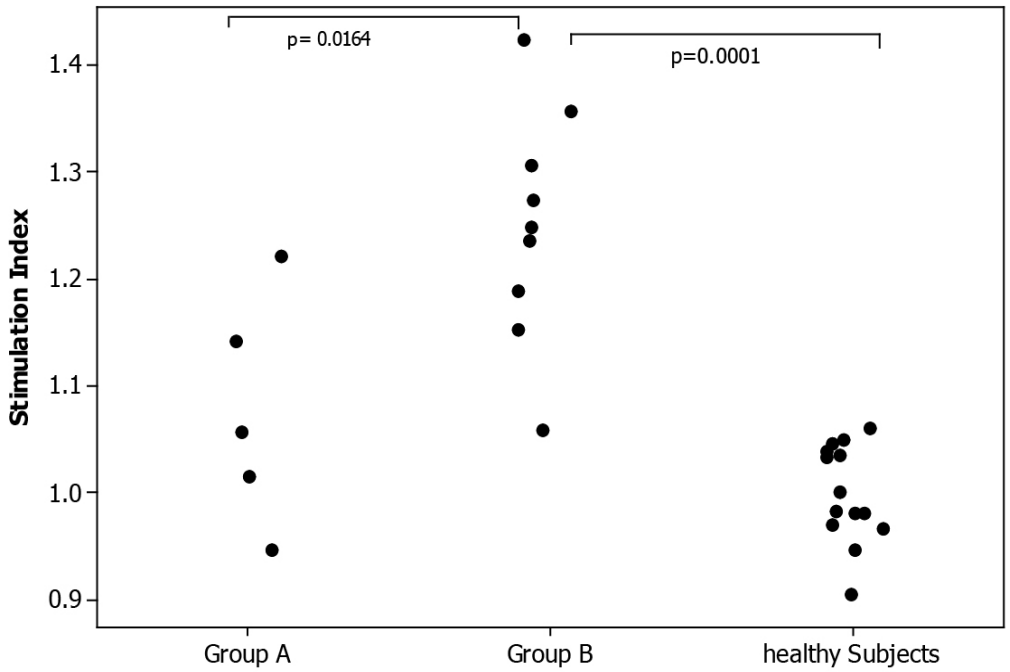

Fig. 2. Lymphoproliferative response to a crude extract from *Gnathostoma binucleatum* in patients clinically diagnosed with gnathostomiasis. Group A, patients at <6 months, and Group B, patients at >6 months after diagnosis; healthy subjects as a control group. Lymphoproliferation was determined by MTT assay. The graph shows the individual IE values for each group. Statistical analysis was done using the Mann-Whitney U test.

patients (p = 0.0164), but one similar to that observed in the group of healthy subjects (p = 0.1513). Also, patients in group B showed a higher lymphoproliferative response than healthy subjects (p = 0.0001).

No differences in the response to *G. binucleatum* antigens between males and females in control and patients groups were found (p = 0.9247, and 0.1066, respectively).

These data suggest that the patients developed a long-lasting, non-protective immune response against the parasite, and that this response was higher approximately 6 months after infection.

This characterization of the immune response to *G. binucleatum* could provide data that help understand the immunological mechanisms involved in the response to this parasite. There are no reports on the cellular response to antigens from *G. binucleatum* in patients, though Somthana *et al*. (2011), reported that partially purified protein antigens from *G. spinigerum* were able to induce the production of antibodies *in vitro*. They found that PBLC from patients showed a significant increase in gene expression related to an innate immune response and decreased cell-mediated immunity, but that the expression of gene-related antibody production was not pronounced (Somthana *et al*., 2011). Although the humoral immune response to *G. binucleatum* has been extensively studied because of its possible use in diagnostics, the cellular immune response reported here suggests that exposure to *G. binucleatum* antigens could be capable of inducing immunological memory. This response, however, is not protective, as shown by the re-incidence of infection upon a second exposure to the larvae.

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