MODULATION BY *POLYPODIUM LEUCOTOMOS* EXTRACT OF CYTOKINE PATTERNS IN EXPERIMENTAL TRICHOMONIASIS MODEL

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Summary:

The immunomodulating effects of Anapsos®, an aqueous hydrosoluble extract obtained from the rhizomes of the fern *Polypodium leucotomos*, on both pathogenicity and cytokine levels in serum (IFN- γ /IL-4) were assayed in a *Trichomonas vaginalis* experimental model (BALB/c mice infected with 10^7 trichomonads and examined at day 15 after infection). Doses of 20 mg/kg/day administered for 10 days before the infection with the parasite induced a decrease of the experimental pathogenicity approximately 10-20 % compared to controls. Gross histopathologic changes at abdominal organs and mortality rate, as a consequence of pathogenicity of the protozoa and the immune response of the host, were evaluated. IFN- γ and IL-4 cytokines were determined on days -5, 0, 5, 10, and 15 postinfection by indirect ELISA. Treatment with PAL before infection modulates and downregulates the IFN- γ concentration, while anticipates and upregulates the IL-4 level. The assays performed have showed the utility of the murine model of experimental trichomoniasis for the evaluation of immunomodulatory activity of synthetic or natural products.

KEY WORDS : *Trichomonas vaginalis*, *Polypodium leucotomos*, Anapsos®, experimental trichomoniasis model, immunomodulation, IFN- γ , IL-4.

Résumé : MODULATION DU PROFIL DES CYTOKINES DANS UN MODÈLE DE TRICHOMONASE EXPÉRIMENTALE PAR UN EXTRAIT DE *POLYPODIUM LEUCOTOMOS*

La modulation de la réponse immunitaire dans un modèle de trichomonase expérimentale chez la souris BALB/c après le traitement avec un extrait de *Polypodium leucotomos* (Anapsos®) a été caractérisée par le niveau des cytokines de type Th1 (IFN- γ) et Th2 (IL-4) du sérum, et aussi par l'activité pathogène de *Trichomonas vaginalis* (10^7 trichomonades inoculés par voie intra-péritonéale) dans la cavité abdominale et la mesure quantitative des lésions dans le péritoine, l'estomac, le pancréas, et le foie, ainsi que la mortalité des souris avant le quinzième jour après l'infestation. Une dose de 20 mg/kg/jour administrée pendant 10 jours avant l'infestation avec les parasites induit une réduction de la pathogénie expérimentale de 10-20 % par rapport au groupe contrôle. Les 5^{ème}, 10^{ème} jours avant l'infestation et les 5^{ème}, 10^{ème}, et 15^{ème} jours après l'infestation ont été déterminés les niveaux de production des cytokines IFN- γ et IL-4 par ELISA indirect. Le traitement avec Anapsos® avant l'infestation induit une réduction importante de la concentration d'IFN- γ , tandis que les niveaux d'IL-4 s'élèvent.

MOTS CLÉS : *Trichomonas vaginalis*, *Polypodium leucotomos*, Anapsos®, trichomonase expérimentale, immunomodulation, IFN- γ , IL-4.

Trichomonas vaginalis is a parasitic protozoan that is the cause of trichomoniasis, the most common sexually transmitted diseases (STD) in the world (Thomason & Gelbart, 1989). The disease encompasses a broad range of symptoms from a state of severe inflammation and irritation with a frothy malodorous discharge to a relatively asymptomatic carrier state (Catterall, 1972). The parasite is a major cause of vaginitis, cervicitis and urethritis in women and may cause nongonococcal urethritis, prostatitis, and perhaps other lower genitourinary tract syndromes in men (Krieger *et al.*, 1990). As a STDs, trichomoniasis could play a major role as a cofactor (Laga *et al.*, 1993) in the transmission of HIV infection.

At present, little is known about precise determinants of virulence among trichomonads or about various key areas of this parasite-host interaction which would help in our understanding of possible mechanisms of

disease pathogenesis. The host-parasite relationship is very complex, and the broad range of clinical symptoms cannot likely be attributed to a single pathogenic mechanism.

On the other hand, Anapsos® is an aqueous extract of the rhizome of the fern *Polypodium leucotomos*. The fern is found in northern Honduras and other parts of Central America. Clinical effects have been described in diseases where immunological defects may play a role such as neoplasm (Horvath *et al.*, 1967), atopic dermatitis (Jiménez *et al.*, 1987), psoriasis (Thomas-Barry, 1999) and vitiligo (Mohammad, 1989).

In the present paper, the application of an experimental trichomoniasis model (Nogal Ruiz *et al.*, 1997) to study iberoamerican plant extracts has allowed us to investigate the foundations of induced immune response in host-parasite interaction, and the immunomodulating effect of Anapsos®. Such activity was measured by the capacity of vegetal extract to diminish or clear abdominal lesions (or mortality) in animals, and by the modulation of immune response, via the elucidation of cytokine patterns (IL-4/IFN- γ).

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MATERIALS AND METHODS

PARASITE AND CULTURE

Trichomonas vaginalis, strain C1-NIH (reference no. 30001), was obtained from the American Type Culture Collection (Rockville, Maryland, USA). The clinical findings of trichomoniasis caused by C1-NIH strain indicates its pathogenicity and it seems to be appropriate to standardize on murine model of experimental trichomoniasis. Parasites are maintained in our laboratory by cryopreservation in liquid nitrogen.

For infecting animals, organisms were grown at 37°C with 5 % CO₂ in Diamond medium (Trypticase-Yeast extract-Maltose) pH 6.2, supplemented with 10 % heat inactivated horse serum, penicillin G (100 IU/ml) and streptomycin (100 mg/ml) (Diamond, 1957). After two or three reseeds in fresh culture medium, enough number of parasites were obtained to infect the animals.

MICE

BALB/c mice from CRIFFA (Barcelona, Spain) bred under standard conditions in our laboratory were used for experimental infections. All animals were maintained in a temperature and humidity controlled environment with a 12 h light/dark cycle and water and food *ad libitum*. BALB/c mice weighed 20 g at the beginning of the experiments.

EXPERIMENTAL DESIGN

One experimental group was infected, but not treated (control group), and another was treated intraperitoneally (experimental group) with 10 Anapsos® doses (20 mg/kg/day), on days 1-10 before infection. To inoculate the animals, logarithmic phase *T. vaginalis* from axenic cultures resuspended in fresh medium were administered in a single dose. Groups of 10 mice were infected intraperitoneally with 10⁷ trichomonads.

PATHOGENICITY DETERMINATION

The pathogenicity index (PI) was determined following the criteria recommended by Toyos (1974) and revised by Nogal Ruiz *et al.* (1997). Briefly, mortality (0-50 points), ascites (0-6 points) and the gross damage produced to the peritoneum (0-10 points), to the spleen/pancreas/stomach (0-12 points), and to the visceral (0-10 points) and diaphragmatic (0-12 points) liver faces were rating according to Table I. Both the mice that succumbed to the infection and those killed under ether anaesthesia at day 15 postinfection were examined at necropsy for the presence of trichomonads

Pathologic manifestations	Assigned values
• Mortality (days p.i.)	
3°	50
4°	44
5°	38
Every delay day	discount 6 points
> 10°	6
• Ascites	
< 1 ml	2
1-2 ml	4
1 ml	6
• Peritoneum	
1-3 SN	2
Multiple SN and/or N1	4
Multiple N1 and/or N2	6
MN affecting pelvis	8
MN affecting intestine and kidneys	10
• Spleen/pancreas/stomach	
1-3 SN and/or splenomegaly	2
Multiple SN and/or N1	4
N2	6
N3 affecting more than one organ	8
N4	10
N5 affecting liver and intestine	12
• Visceral liver side	
1-3 SN	2
N1	4
N2	6
N3	8
N4	10
• Diaphragmatic liver side	
1-3 SN and/or colour changes	2
N1	4
N2	6
N3	8
N4	10
N5	12

SN: single necrosis; N1: necrosis of 3-5 mm diameter; N2: necrosis of 5-8 mm; MN: multiple necrosis; N: necrosis; N3: necrosis of 8-10 mm; N4: necrosis of 10-15 mm; N5: necrosis of more than 15 mm of diameter affecting the most organ surface.

Table I. – Rating of pathological manifestations in mice infected by the intraperitoneal route with *T. vaginalis* (Nogal Ruiz *et al.*, 1997).

and for gross-pathologic changes in the abdominal cavity. PI was then calculated as the arithmetic mean of the values for each parameter, ranging from 0 to 100.

STATISTICAL ANALYSIS

Statistical analysis was performed using the non-parametric Mann-Whitney U test. A probability $p < 0.05$ was considered indicative of statistical significance.

PREPARATION OF ANAPSOS® (POLYPODIUM LEUCOTOMOS EXTRACT)

Polypodium leucotomos rhizomes were harvested in the Experimental and Ecological Recuperation Plantations (property of ASAC Pharmaceutical Interna-

tional, AIE) in Guatemala, located at 2,000 m above sea level. Once their identification was verified by botanists from the University of San Carlos in Guatemala, the rhizomes were dehydrated at 50° C for 48 h. After grinding, the rhizomes were extracted by maceration and percolation with a mixture water-alcohol at 60° C for 48 h. After the extraction, the product was purified by adsorption chromatography and ion-exchange chromatography A column Supercosil LC-18 (25 cm × 4.6 mm × 5 mm) and solvent system (0.3 % v/v phosphoric acid in water-water-acetonitrile) with a flow rate 0.85 ml/min was used. The detection was carried out by the determination of absorbance samples at 210 nm. The final product was filtered-freeze dried in 50 mg vials and granted by ASAC for the present research work.

SOURCE OF SERUM

BALB/c mice were bled under anaesthesia, from the retroorbital plexus, on days - 5, 0, 5, 10, and 15 postinfection. Blood from each group was pooled and centrifuged to obtain sera. Different serum samples were harvested and frozen at - 20° C.

ELISA FOR THE DETERMINATION OF MURINE IL-4 AND IFN- γ

Enzyme-linked immunosorbent assay for the quantitative measurement of mouse IL-4 and IFN- γ in serum (96-well microtitre plates) was performed with ENDOGEN® (ENDOGEN Inc., Woburn, Massachusetts, USA). The results were calculated using curve fitting statistical software (SPSS/PC + Release 10.0.6., SPSS Inc., Chicago, Illinois, USA).

RESULTS

PATHOGENICITY DETERMINATION

Values assigned to the pathological manifestations (Fig. 1a) and pathogenicity indices (PI) of *T. vaginalis* (Fig. 1b) were determined at necropsy (day 15 postinfection) in mice BALB/c treated with 10 preinfection Anapsos® doses (20 mg/kg/day), and infected with 10⁷ trichomonads (ANP group) against control group (this group was infected and not treated). When Anapsos® was administered a decrease of experimental pathogenicity was observed, approximately 10-20 % compared to controls. PI of ANP group presents significant differences statistically against PI of control group according to U Mann-Whitney test.

ELISA FOR THE DETERMINATION OF MURINE IL-4 AND IFN- γ

In order to find a molecular evidence of the immunomodulating effect of Anapsos®, determination of murine IL-4 (Fig. 2a) and IFN- γ (Fig. 2b) in serum by ELISA with ENDOGEN® were performed.

BALB/c mice infected intraperitoneally with *T. vaginalis* (control group) suffer a dramatic increase of IFN- γ concentration. Maximum level (6,825 pg/ml) of this cytokine was reached on day 10 postinfection (pi). On the other hand, also a weak increase of IL-4 was showed in control group, with 43 pg/ml (day 10 pi). However, in murine model of experimental trichomoniasis, Anapsos® preinfection treatment of 10 days (ANP group), modulates and downregulates the IFN- γ concentration with 358 pg/ml (day 10 pi). Likewise,

Fig. 1. – Values assigned to the pathologic manifestations after 15 days in BALB/c mice treated with Anapsos® (20 mg PAL/Kg/day × 10), and infected with 10⁷ *T. vaginalis* (A), and mean pathogenicity indices (B), as expressed by the arithmetic means and the standard deviation from at least three values obtained in separate experiments. The asterisk shows differences statistically significant in comparison with untreated control group by Mann-Whitney U test ($p < 0.05$).

CTRL: control group; ANP: ANP group; M: mortality; A: ascites; P: peritoneum; S/P/S: spleen/pancreas/stomach; L(V): liver (visceral face); L(D): liver (diaphragmatic face); P.I.: pathogenicity index.

Fig. 2. – Dynamics of cytokine responses in sera from BALB/c mice treated with Anapsos® (20 mg/Kg/day $\times$ 10), and infected with *T. vaginalis*: IL-4 (A) and IFN- γ (B) levels in ANP (■) and control (◆) groups. Each point represents the mean optical density values $\pm$ standard deviation of six data for duplicate.

Anapsos® treatment anticipates and upregulates the IL-4 level in serum with 46 pg/ml on day 5 pi compared with control group. On day 15 postinfection, the cytokines were diminished to baselines in both groups.

DISCUSSION

At present, little is known about the pathobiochemistry of the trichomoniasis. Modern research has focused on the initial events required to establish infection (Petrin *et al.*, 1998). Many mechanisms are thought to be involved and include cell-to-cell adhesion (Alderete *et al.*, 1995), haemolysis (Fiori *et al.*, 1993) and the excretion of soluble factors such as extracellular proteinases (Arroyo *et al.*, 1989), and cell-detaching factor (Garber *et al.*, 1989). Like many other protozoa, trichomonads displays phenotypic variation as a mechanism of immune evasion (Alderete *et al.*, 1995). Also, *T. vaginalis* secretes copious amounts of highly immunogenic soluble antigens, a continuous release of these antigens may neutralize antibody or cytotoxic T lymphocytes, and numerous cysteine proteinases that degrade IgG, IgM, which allows the organism to survive the antibody response. As well, this flagellated protozoa can coat itself with host plasma proteins. This coating does not allow the host immune system to recognize the parasite as foreign.

Many animal models have been proposed for studying of the pathogenesis of *T. vaginalis* infection (Kulda, 1990). Of particular importance were intraperitoneal assays (Teras & Roigas, 1966; Cavier *et al.*, 1972). In our experiments, intraperitoneal inoculation of *T. vaginalis* into mice causes fibropurulent peritonitis with abscesses and necrotic foci in abdominal organs (especially pancreatic and hepatic) or phlegmonous inflammatory

changes and production of ascitic fluid whose extent is proportional to the level of virulence of the inoculated strain (Nogal Ruiz *et al.*, 1997).

Most of inoculated parasites are destroyed by non-specific cytotoxic activity of peritoneal cells shortly after they are introduced into the abdominal cavity. An infective foci including a large number of neutrophils and a small number of macrophages can be seen during the first 48 h at the site of infection. After 48–72 h, the neutrophils are replaced with activated macrophages which would be able to ultimately clear the infection (Kulda, 1990).

The surviving trichomonads retreat to abdominal organs (pancreas, liver, mesentery lymph nodes) establishing isolated foci in which the parasites multiply. Subsequently, the infection spreads from these foci by development and progressive extension of tissue lesions. A characteristic feature of the more advanced infection is the penetration by necrotic caseous matter of the liver, pancreas, stomach, and spleen, the viscera are covered also with fibropurulent exudates (Nogal Ruiz *et al.*, 1997). As the infection progresses, the volume of the ascitic fluid increases (> 3 ml/mouse). The final phase of infection is a generalized fibrinopurulent peritonitis, which can result in death.

On the other hand, approaches to immunomodulator effects of Anapsos® have been attempted in animal models infected by parasites. In our experience confirmed by *in vitro* (Sempere *et al.*, 1997) or *in vivo* (Cuéllar del Hoyo *et al.*, 1997; Dea Ayuela *et al.*, 1999) observations, Anapsos® immunomodulating effect is only shown in the presence of an immunological stimulus. Cuéllar del Hoyo *et al.* (1997) have studied the biological effect of this vegetal extract in BALB/c mice immunized with the third larval stage antigens of *Anisakis simplex*. A decrease in the humoral response (specific IgG1

isotype) was observed. Likewise, IgG2a subclass antibody levels were higher than those of the untreated group. The results from this study seem to indicate that extract affects the Th1/Th2 balance. Moreover, Dea Ayuela *et al.* (1999) have investigated the immunomodulant effects of Anapsos® in BALB/c mice immunized with a larvae (L1) extract of *Trichinella spiralis*. Suppression of the IgG1 response was transient, as serum levels were significantly decreased and potentiates the IgG3 response against *T. spiralis*. This helminth model seem to indicate that extract downregulates the Th2 responses generated against *T. spiralis* antigen.

Elucidation of immunomodulator response of extract in experimental model of *T. vaginalis* have been limited to differential evaluation of gross histopathologic changes and mortality rate of infected mice. In our experiments, when Anapsos® was administered at doses of 20 mg/kg/day for 10 days before infection, the PI decrease approximately 10-20 % compared to controls (see Fig. 1b). Likewise, in order to confirm this immunomodulating effect the determination of murine IL-4 and IFN- γ were performed. This extract modulates and downregulates the IFN- γ concentration, and anticipates and upregulates the IL-4 level in serum.

Biological interpretation about the role of both cytokines is very elucidating. IFN- γ is capable of regulating specific immune effector mechanisms by direct actions on helper T cells, cytotoxic T cells, and B cells, and it augments their microbicidal activities, such mechanisms are induced by inflammatory mediators (TNF- γ , IL-1, IL-6, and IL-8). IFN- γ augments the induction of Th1 cells, that regulates cell-mediated immunity (Swain *et al.*, 1991), and impedes the induction, proliferation and effector functions of Th2 cells, that regulate many components of humoral immunity (Gajewski *et al.*, 1989). Also, IFN- γ induce transendothelial migration of inflammatory cells (Oppenheimer & Ziff, 1988), this is reflected by their ability to induce delayed-type hypersensitivity (DTH) responses. During DTH reactions, two cell types are specifically recruited to the site of the cession, T-helper lymphocytes and cells of the monocyte/macrophage lineage. Their *in situ* accumulation leads to the formation of granulomas. The ability of IFN- γ to evoke phagocyte-mediated defense against infections by enhancing the ability of macrophages to phagocytose (Voyich *et al.*, 2001), and subsequently destroy microbes (Abbas *et al.*, 1996) suggests that this effector mechanism could destroy *T. vaginalis*.

On the other hand, IL-4 plays a critical role in immunoglobulin class-switching (Schultz & Coffman, 1992). Also, this cytokine has potent regulatory effects on the differentiation of both helper and cytolytic T lymphocytes. IL-4 augments the induction of Th2 cells (humoral immunity, eosinophilia and mastocytosis) which plays a major role in regulation of many components of humoral immunity (Swain *et al.*, 1990). IL-4 inhibits

the release of the inflammatory mediators TNF- α , IL-1, IL-6, IL-8, TGF- β (Fiorentino *et al.*, 1991), and prostaglandin E₂ (PGE₂) from activated monocytes, and can inhibit DTH responses *in vivo*. Parasite killing is most effectively stimulated by the Th1 cytokine IFN- γ , and is inhibited by several Th2 cytokines, including IL-10, IL-4 and IL-3 (Liew *et al.*, 1989). Similar correlations between disease outcome and cytokine profile have been established for a variety of rodent and human diseases, including bacterial, mycobacterial, fungal (Romani *et al.*, 1992), and helminthic (Scott *et al.*, 1989; Urban *et al.*, 1992) diseases.

In conclusion, in murine model of experimental trichomoniasis, a) the presence of both IFN- γ and IL-4 in serum of the control group (untreated and infected mice) suggests that both Th1 and Th2 cells are active, although with a Th1-dominant response during the early stages after *T. vaginalis* infection, b) the immunological environment observed in murine abdominal lesions is compatible with a inflammatory phenomenon of delayed-type hypersensitivity regulated by Th1 cells fundamentally, with lesser contribution of Th2 cells, and c) Anapsos® (*Polypodium leucotomos* extract) has showed a immunomodulator activity *sensu stricto*, the cytokine patterns (IFN- γ /IL-4) of mice treated and infected (ANP group) seem indicate that extract affects the Th1/Th2 balance.

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