Immunotherapy for pythiosis: Effect on NTPDase activity in lymphocytes of an experimental model

Barbara Charlotte Bach, Daniela Bitencourt Rosa Leal, Jader Betsch Buchel, Viviane do Carmo Gonçalves Souza, Grazieli Maboni, Marcelo Dal Pozzo, Karine Bizzì Schlemmer, Sydney Hartz Alves, Janio Morais Santurio*

Departmento de Microbiologia e Parasitologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Prédio 20, 97105-900 Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 25 May 2010
Accepted 10 September 2010

Keywords:
NTPDase
Pythium insidiosum
Rabbit lymphocytes

ABSTRACT

NTPDase (EC 3.6.1.5) occurs in lymphocytes and plays an important role in immune function, in that it hydrolyzes extracellular nucleoside tri- and/or diphosphates to form AMP. Pythium insidiosum causes the disease pythiosis, a pyogranulomatous disease of horses, dogs, cattle, cats and humans. Most antifungal drugs are ineffective against this pathogen, and immunotherapy, a treatment approach that relies on the injection of P. insidiosum antigen, has been successfully used in humans and horses to manage this disease. In this study, we investigated NTPDase activity in lymphocytes from rabbits inoculated with zoospores of P. insidiosum. After immunotherapy, we investigated the relationship between enzymatic activity and the pattern of the immune response. One milliliter of zoospores was inoculated subcutaneously into the coastal region of each rabbit. An average of 17,500 viable mobile zoospores/mL of induction medium was administered. Inoculated rabbits were checked weekly, and the subcutaneous nodular area (cm²) was measured 28 days after inoculation. Rabbits that developed lesions received eight doses of immunotherapy at intervals of 14 days. Blood samples were collected by heart puncture twice a month for the determination of NTPDase activity. The results demonstrated that NTPDase activity in lymphocytes was increased in relation to ATP hydrolysis (by about 100%) in pythiosis and returned to normal values after immunotherapy. The data demonstrating NTPDase activity before and after immunotherapy reinforce the previously elaborated hypothesis that the changes from a Th2 to a Th1 immune response is responsible for the curative properties of immunotherapy.

© 2010 Published by Elsevier Masson SAS.

1. Introduction

Enzymes that hydrolyze extracellular nucleotides are known as ectonucleotidases, because they are anchored to the cell surface with their active site facing the extracellular medium [1]. NTPDase (EC 3.6.1.5) is an enzyme that hydrolyzes extracellular nucleoside tri- and/or diphosphates (preferably ATP and ADP) and is found in many cell types, such as lymphocytes and platelets [2–4]. The AMP that is produced is converted to adenosine by the catalytic action of ecto-5’-nucleotidase enzyme (EC 3.1.3.5). NTPDase and 5’-nucleotidase control the levels of two potent immunomodulatory molecules, ATP and adenosine. ATP acts as a pro-inflammatory agent that potentiates the release of pro-inflammatory cytokines [5] from activated lymphocytes [6]. Adenosine, in contrast, exhibits potent anti-inflammatory and immunosuppressive action by inhibiting the proliferation of T cells [7], the secretion of cytokines and the migration of leukocytes across endothelial barriers [8].

NTPDase activity occurs in lymphocytes from human peripheral blood, thymi and mouse spleens, and in humans, its activity is higher in B cells than in T cells [9]. The possible association between NTPDase activity and immune diseases has been evaluated in humans [10], considering that NTPDase activity could be used as an activation marker of lymphocytes during the immune response.

The oomycota Pythium insidiosum is the cause of the disease pythiosis, a chronic, pyogranulomatous disease of horses, dogs, cattle, cats and humans [11]. Rabbits are sensitive to zoospore inoculation and can be used as experimental models for studying the disease [12]. The hyphae of this microorganism colonize cutaneous and subcutaneous tissues, produce intestinal lesions, invade blood vessels, and proliferate within bone. Lacking the membrane steroid ergosterol, P. insidiosum is unaffected by the usual battery of antifungal agents, and immunotherapy shows promise in the management of infections in horses [13]. In the infected host, P. insidiosum triggers a T helper 2 (Th2) response with an inflammatory reaction composed mainly of eosinophils...
and mast cells. A switch from a Th2 to a Th1 response is postulated to be the most likely explanation for the curative properties of immunotherapy [14].

The aim of this study was to determine whether immunotherapy affects NTPDase activity in lymphocytes from rabbits with pythiosis, with the ultimate goal of assessing the potential use of NTPDase activity as a peripheral marker of immune function in pythiosis infection.

2. Materials and methods

2.1. Materials

Nucleotides and Trizma base were purchased from Sigma (St. Louis, MO, USA). Ficoll-Hypaque (Lymphoprep™) was purchased from Nycomed Pharma (Oslo, Norway). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Pythium insidiosum

DMVP 118/98 (CBS 101555) strain, isolated from a pythiosis lesion in the chest of a foal and cultivated in 1.5% Corn Meal Agar at 27 °C for 7 days, was used to experimentally inoculate rabbits and to produce the immunotherapy.

2.3. Zoosporogenesis

Ten pieces of Paspallum notatum grass, approximately 2 cm long, were autoclaved for 2 hours and distributed over 68 P. insidiosum cultures and incubated at 37 °C for 24 hours. Pieces of infected grass were transferred to an induction medium that contained the following: solution 1, 1.0 M K2HPO4, 1.0 M KH2PO4, 3.66 M (NH4)2, 500 mL of distilled water; solution 2, 0.5 M MgCl2 6H2O, 0.5 M CaCl2 2H2O, 250 mL of distilled water. The final composition of the induction medium was 0.5 mL of solution 1 and 0.1 mL of solution 2 in 1000 mL of sterile distilled water. Induction medium containing grass infected by P. insidiosum was incubated at 37 °C for 8 hours. Pieces of grass were observed by microscopy, and zoospores were counted using a Neubauer chamber.

2.4. Rabbits

Forty “New Zealand” male rabbits, aged between 2 and 3 months, were used in this work. One milliliter of zoospores was inoculated subcutaneously into the coastal region of each of 20 rabbits. An average of 17,500 viable mobile zoospores/mL of induction medium was administered. Inoculated rabbits were checked weekly, and the subcutaneous nodular area (cm²) was measured using a sliding calliper when nodules were present. Nodular development was evaluated 28 days after inoculation. Those rabbits not developing lesions were eliminated from the experiment after 28 days. ELISA was used to confirm Pythium infection [15]. Rabbits that developed lesions received eight doses of immunotherapy or placebo at intervals of 14 days, beginning 1 month after inoculation with viable mobile zoospores. Blood samples were collected by heart puncture twice a month for the determination of NTPDase activity.

2.5. ELISA

Antibodies against P. insidiosum were measured by ELISA for serodiagnosis of pythiosis, as previously described by Santurio et al. [15]. The antigen used for the immunization of the rabbits, it was prepared of cultures of P. insidiosum stains CBS 101555. The samples of this Oomycete were cultivated under agitation in Sabouraud broth at 37 °C for 7 days. After the cultivation, it was filtered and the mycelia diluted in solution of PBS and sonicated until disruption and centrifuged at 6000 rpm for 5 minutes to collected the supernatant to use as antigen. Plates of polyestrene of 96 wells were sensitized with the antigen diluted in PBS and was incubated to 4 °C for overnight that there was the absorption of the antigen in the surface of the plate. Each well received 10 μg of proteins and, after the incubation period, the plates were washed and stored 4 °C until the moment of the use. The tested sera were diluted in PBS in pH 7.2 (1:2000), distributed 100 μl in each well in the plates and incubated at 37 °C for 1 hour. Then, the plates were incubated with specific secondary antibody for the species (anti-IgG conjugated with peroxidase) with dilution of 1:10,000. The plates received the chromogen buffer (ortho-phenylene-diamine) and the reading was carried out through a spectrophotometer of plates with 490 nm.

2.6. Immunotherapy

P. insidiosum was cultivated in 150 mL of Sabouraud broth incubated at 37 °C in a shaker at 130 rpm for 8 days. After removing the culture from the shaker, mycelial material was inactivated with 0.02% thimerosal for 20 minutes. The mycelial mass was disrupted by vortexing at 1800 rpm for 5 minutes, mixed with sulfuric ether ((C2H5)2O) at 50% volume of the mass and rested for 15 minutes before disrupting for 5 minutes more, according to the protocol described by Santurio et al. [16]. The final product was storage in 2 ml aliquots in glass bottle with a rubber lid and lyophilized for 12 hours. Rabbits were inoculated with zoospores. The first dose of immunotherapy was administered subcutaneously on the 30th day after inoculation and in the coastal region opposite to the zoospore injection. This procedure was repeated every 14 days for a total of four injections.

2.7. Isolation of mononuclear cells from human blood

Mononuclear leukocytes were isolated from human blood collected with EDTA and separated on Ficoll-Hypaque density gradients as described by Böyum [17].

2.8. Enzyme assays

After the isolation of mononuclear cells, NTPDase activity was determined by a colorimetric assay in compliance with Leal et al. [10]. The reaction medium contained 0.5 mM CaCl2, 120 mM NaCl, 5 mM KCl, 60 mM glucose, and 50 mM Tris-HCl buffer, pH 8.0 at a final volume of 200 mL. Twenty microliters of intact mononuclear cells suspended on saline solution were added to the reaction medium (2–4 mg protein) and preincubated for 10 minutes at 37 °C. The reaction was started by the addition of substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 mL 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. The samples were chilled on ice for 10 minutes before assaying for the release of inorganic phosphate (Pi) as described for Chan et al. [18], using malachite green as a colorimetric reagent and K2HPO4 as standard. All samples were run in duplicate or triplicate and specific activity is reported as nmol Pi released/min/mg protein.

2.9. Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard, as described by Bradford [19].

2.10. Cellular integrity

The activity of lactate dehydrogenase (LDH) was used as a marker of cell integrity. The measurement of LDH activity showed...
that most cells (approximately 85%) were intact after the isolation procedure (data not shown). The integrity of the cells after incubation was confirmed by microscopic observations in control samples and samples with therapeutic doses (data not shown).

2.11. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test, using a level of significance of 5%.

3. Results

After 28 days, 20 rabbits did not develop lesions. Ten rabbits developed lesions and received four doses of the immunotherapy, as described in “Materials and Methods”. Rabbits with lesions characteristic of pythiosis showed alterations in ATP hydrolysis. Compared to the control (28.5 nmol P/min/mg protein; SEM = 3.77; n = 10), enzyme activity was significantly higher in the group with lesions (60.7 nmol P/min/mg protein; SEM = 5.59; n = 10; P < 0.001). In the inoculated animals that showed no lesions after 28 days, the enzyme activity was similar to control animals (34.5 nmol P/min/mg protein; SEM = 2.92; n = 20). Post-hoc analysis with the Tukey-Kramer test revealed that the group with lesions was significantly different compared to the control group and the group without lesions (Fig. 1).

ATP hydrolysis was also evaluated 14 days after inoculation (37.8 nmol P/min/mg protein; SEM = 8.12; n = 10) (Fig. 2). The results showed a progressive increase in enzymatic activity, but the ATP hydrolysis was significantly higher after 28 days (60.7 nmol P/min/mg protein; SEM = 5.59; n = 10; P < 0.01). Compared to inoculation day (day 0), the Tukey-Kramer test revealed that ATP hydrolysis was significantly different only at day 28. After four doses of immunotherapy, the enzymatic activity returned to levels similar to those observed on the day of inoculation (28.3 nmol P/min/mg protein; SEM = 3.44; n = 10) (Fig. 3). The decrease in enzymatic activity with immunotherapy was statistically significant by the Tukey-Kramer test (P < 0.01). Significant alterations in ADP hydrolysis were not observed after inoculation or immunotherapy (data not shown).

4. Discussion

Much remains to be discovered about the pathogenesis of P. insidiosum infection and the type of immune response elaborated by the host. Most antifungal drugs are ineffective against this pathogen, and immunotherapy, a treatment approach that relies on the injection of P. insidiosum antigen from in vitro cultures, has been successfully used in humans and horses to manage this disease. Mendoza et al. [14] speculated that although key cytokines are up-regulated after successful immunotherapy, a mononuclear reaction is also immediately stimulated by the immunotherapy in infected hosts. It is quite possible that the immunotherapy triggers a cell-mediated response, including natural killer cells and cytotoxic lymphocytes that are abundant in the infected tissues after immunotherapy [20,21], thus compensating for the deficit of phagocytic cells in this population of individuals. A switch from a Th2 to a Th1 response is postulated as the most likely explanation for the curative properties of this approach [22].

Massive leakage of nucleotides might occur upon cell lysis; however, this non-specific mechanism is restricted to organ injury, traumatic shock or certain inflammatory conditions [5]. NTPDase/CD39 is important for the removal of ATP and the coordinate regulation of immune responses [23]. The increase in extracellular ATP levels drives inflammatory dendritic cell and macrophage differentiation, accompanied by the release of pro-inflammatory mediators (TNF-α, IL-12, IL-1β and ROS), the differentiation of naive Th cells to Th1 lymphocytes and the initiation of inflammation. When immune cells are intermittently exposed to low ATP levels, an ATP-sensing mechanism is activated and the cell surface CD39 receptor is up-regulated. This mechanism might be the core of the NTPDase/CD39 enzymatic activity, which is expressed on the surface of cells involved in the immune response.
concentrations, the evolving immune response skews towards tolerance and non-responsiveness rather than cellular reactivity [24].

High local concentrations of ATP accumulating in the immunological synapse between effector cells and target cells can activate pore-forming P2X7 receptors expressed on target cells, resulting in target cell lysis [25–27]. A physiological function for P2X7 remained elusive until more recent studies showed a role for P2X7 in promoting pro-inflammatory responses and controlling intracellular infection in vitro. Several pathogens are now known to protect infected cells against P2X7-dependent apoptosis by producing ATPases that consume extracellular ATP [28].

It appears that low-level purinergic signaling by P2 receptors, induced by nucleotides at decreased concentrations, modulates ongoing inflammatory and immune responses. Activation of P2 receptors, most likely P2Y receptor subtypes, attenuates pro-inflammatory cytokine production by Mo/Mφ, diminishes the Th1 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions. Thus, upon progression of the immune response, nucleotide-mediated purinergic signaling may switch from being predominantly pro-inflammatory to being mostly immunomodulatory, depending on the extracellular concentrations of the nucleotides as well as the P2 receptor subtype(s) ligated by these nucleotides. This switch could be part of a mechanism for fine-tuning the immune effector class in response to microenvironmental signals according to the tissue in which the response occurs [29–31]. Furthermore, Ado-mediated P1 receptor signaling during the second stage down-regulates neutrophil effector functions, contributes to alternative activation of macrophages, stimulates the Th2 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions [5]. Conceptually, the idea of ectoenzymatic control of leukocyte trafficking allows new insights into adhesive events and offers multiple novel targets for manipulating the movement of immune cells. ATP-generating and ATP-consuming pathways coexist on the surface of leukocytes and endothelial cells, and their dynamic balance regulates local ATP and adenosine levels in this microenvironment [32].

Our results demonstrate that during pythiosis, there is an increase in the activity of NTPDase in relation to ATP hydrolysis, without alterations in the rate of ADP hydrolysis. As a result, we expect that there will be a decrease in the concentration of extracellular ATP. At low concentrations, extracellular ATP, as well as ADP, possesses an affinity for the P2Y purinergic receptors on the surfaces of lymphocytes. Binding to the P2Y receptor stimulates the Th2 immune response, leading to the production of IL-4 and activation of eosinophils and mast cells, which can ultimately cause significant tissues lesions. After treatment with four doses of immunotherapy, we observed that the rate of ATP hydrolysis was similar to the rate observed prior to the inoculation. Thus, the decrease in NTPDase activity could be responsible for the increase of the extracellular ATP concentration, which, at high concentrations, could bind to P2X7 receptors. The interaction with purinergic receptors would provoke a Th1 response pattern, with secretion of pro-inflammatory cytokines and activation of cytotoxic T lymphocytes and macrophages that would destroy the hyphae. The switch from a Th2 to a Th1 response pattern would lead to a decrease in the number of lesions (Fig. 4).

5. Conclusion

In conclusion, the observed change in NTPDase activity before and after immunotherapy reinforces the hypothesis, previously elaborated by Mendonza and Newton [22] that the change from a Th2 to a Th1 immune response might be responsible for the curative properties of immunotherapy.

Acknowledgements

The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundo de Incentivo à Pesquisa da Universidade Federal de Santa Maria (FUND/UFSM) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biopha.2010.09.016.

References

[10] Leal DBR, Streher CA, Neu TN, Bittencourt FP, Leal CAM, Silva JEP, et al. Characterization of NTPDase (NTPDase 1; ecto-apyrase; ecto-diphosphohydrolase;...


