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Original article

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

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ABSTRACT

NTPDase (EC 3.6.1.5) occurs in lymphocytes and plays an important role in immune function, in that 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 (cm^2) was measured 28 days after inoculation. Rabbits that developed lesions received four 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 change from a Th2 to a Th1 immune response is responsible for the curative properties of immunotherapy.

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 [2] and platelets [3,4]. 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, thymocytes and mouse spleen lymphocytes, and its activity is higher in human 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

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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 *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 K_2HPO_4, 1.0 M KH_2PO_4, 3.66 M $(NH_4)_2$, 500 mL of distilled water; solution 2, 0.5 M $MgCl_2$ $6H_2O$, 0.5 M $CaCl_2$ $2H_2O$, 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. The control group, with ten rabbits, received one milliliter of saline, while thirty rabbits were inoculated with one milliliter of zoospores subcutaneously into the coastal region. An average of 17,500 viable mobile zoospores/mL of induction medium was administered. Inoculated rabbits were checked weekly, and the subcutaneous nodular area (cm^2) 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 four 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 polystyrene 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 serums 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 spectrophotomer 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% thimersal for 20 minutes. The mycelial mass was disrupted by vortexing at 1800 rpm for 5 minutes, mixed with sulfuric ether ($(C_2H_5)_2O$) 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 rabbit blood

Mononuclear leukocytes were isolated from rabbit 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. [2]. The reaction medium contained 0.5 mM $CaCl_2$, 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 KH_2PO_4 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