Generation and characterization of polyclonal and monoclonal antibodies to human NTPDase2 including a blocking antibody

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Abstract Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2) is an ectonucleotidase that modulates P2 receptor activation by hydrolyzing ATP to ADP. In rodents, NTPDase2 is expressed by several specialized cell types such as vascular adventitial cells, neuroglial cells, hepatic portal fibroblasts, gustatory type I cells, and cells within the connective tissues of reproductive and gastrointestinal organs. Much less is known regarding the expression and function of NTPDase2 in humans. Here, we developed specific research tools to study human NTPDase2. We generated mouse monoclonal antibodies and rabbit polyclonal antibodies specific to human NTPDase2 and validated their specificity by western blot, immunocytochemistry, immunohistochemistry, and flow cytometry. In addition, one monoclonal antibody named hN2-D5_s specifically inhibits human NTPDase2 enzymatic activity but not mouse nor rat NTPDase2. Using these antibodies, NTPDase2 immunoreactivity was detected on glial cells of the human enteric nervous system suggesting a function of the enzyme in intestinal motility. In conclusion, the new antibodies described in our work are novel tools that will enhance

future studies of NTPDase2 expression and function in humans.

Keywords Monoclonal and polyclonal antibodies · Human NTPDase2 · cDNA immunization · Inhibitor

Introduction

Nucleoside triphosphate diphosphohydrolase-2 (NTPDase2) is one of the eight members of the E-NTPDase family (denoted NTPDase1 to 8) [1]. NTPDase1, NTPDase2, NTPDase3, and NTPDase8 are integral proteins of the plasma membrane [2] that work in concert to hydrolyze nucleotides at the cell surface. But each displays different affinities and abilities and these differences result in the differential activation of P2 receptors and associated functions [3, 4]. NTPDase2 hydrolyses triphosphonucleosides such as ATP and UTP efficiently but diphospho- derivatives very poorly [5, 6]. These biochemical characteristics facilitate the activation of nearby ADP- and UDP-transducing receptors such as P2Y₁ and P2Y₆, respectively.

Numerous studies in animal models suggest that NTPDase2 regulates key intercellular signaling processes throughout the body. For example, NTPDase2 is expressed on the surface of vascular adventitial cells and here, it is thought to play a role in vascular integrity by favoring the activation of the ADP receptors P2Y₁ and P2Y₁₂ on platelets [7]. NTPDase2 is also expressed by type 1 cells of taste buds [8] where it participates in taste transduction [9]. Other proposed functions of NTPDase2 include contributing to eye formation in *Xenopus laevis* [10] but not in mouse [11], regulating neuronal development [12] and modulating biliary epithelium proliferation in rat [13]. NTPDase2 is also localized in Bowman's capsules within the kidney but its function there is

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not defined [14]. Although the recent development of NTPDase2 KO mice will facilitate the identification of the functions of NTPDase2 [9], studies of NTPDase2, particularly in humans, are still hampered by the lack of tools such as specific antibodies and inhibitors that can be easily and readily obtained.

Here, we addressed this issue by developing novel and specific monoclonal and polyclonal antibodies to human NTPDase2. We have previously raised polyclonal antibodies to rat [7] and mouse NTPDase2 [8] and used this experience to develop and validate the specificity of the new human NTPDase2 antibodies by several complementary techniques. Interestingly, one of the generated hybridomas produced an immunoglobulin that specifically inhibits human NTPDase2 protein activity. Together, these tools will be a great aid to future research efforts to define the role of NTPDase2.

Materials and methods

Materials

Aprotinin, phenylmethanesulfonyl fluoride (PMSF), sodium citrate, 3,3'-diaminobenzidine (DAB), tris(hydroxymethyl)aminomethane (Tris), and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic solution, and NuPAGE 4–12% Bis-Tris gels were obtained from Life Technologies (Burlington, ON, Canada). Fetal bovine serum (FBS) and goat serum were from Wisent (St-Bruno, QC, Canada). For western blot, immunohistochemistry, and immunocytochemistry experiments, the secondary antibodies used were either conjugated to horseradish peroxidase, goat anti-mouse from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA) or donkey anti-rabbit from GE Healthcare Life Science (Mississauga, ON, Canada), or to biotin, namely goat anti-mouse from Vector Laboratories (Burlington, ON, Canada) and goat anti-rabbit from Jackson ImmunoResearch Laboratories Inc. For flow cytometry experiments Alexa Fluor 633-goat anti-mouse and Alexa Fluor 594-donkey anti-rabbit were obtained from Life Technologies. For whole-mount immunohistochemical labeling, the secondary antibodies used were Alexa Fluor 488-goat anti-rabbit, Alexa Fluor 568-goat anti-rabbit from Invitrogen (Carlsbad, CA, USA), and Alexa Fluor 488-donkey anti-mouse and Alexa Fluor 594-Streptavidin from Jackson Laboratories (Bar Harbor, ME, USA).

Animals and ethical approval

Females BALB/c mice and New Zealand rabbits were obtained from Charles River Laboratories (St-Constant, QC,

Canada). All procedures were approved by the Canadian Council on Animal Care and the Université Laval Animal Welfare Committee. Human intestines were obtained under approved institutional review board protocol (*Comité Éthique de la Recherche du CHU de Québec - Université Laval*) following written consent. Work involving human jejunum whole preparation was approved by the institutional review board of Michigan State University (IRB 13-945M).

Plasmids

The plasmids encoding human NTPDase1 (GenBank accession number U87967) [15], NTPDase2 (NM_001246) [16], a kind gift of Dr. A.F. Knowles (San Diego, CA); NTPDase3 (AF034840) [17], a kind gift of Dr. T.L. Kirley (Cincinnati, OH); and human NTPDase8 (AY430414) [18], mouse NTPDase2 (AY376711) [2] or rat NTPDase2 (NM_172030) [5], a kind gift of Dr. H. Zimmermann (Frankfurt am Main, Germany), were used for antiserum generation and/or for cell transfection, as described below.

Monoclonal antibody production by cDNA immunization

Hybridomas were generated in BALB/c mice injected four times at days 1, 22, 78, and 134 with 30 μ L of the human NTPDase2 expressing plasmid (2 mg/mL) diluted in Hank's balanced salt solution (HBSS). A single intramuscular (IM) injection was made in the tibialis anterior. An electrode cream was applied on the skin and electroporation was carried out at the site of DNA injection with two electrode plates connected to an electroporator according to the following parameters: seven pulses of 100 $V \cdot cm^{-1}$ and duration of 20 ms. A final injection was made at day 204 with 14 million intact human embryonic kidney (HEK 293T) cells transfected with the human NTPDase2 expression vector. Spleen cells were collected 3 days after the final injection and fusion with SP2/0 cells were done as previously described [19] with the following minor modification: the SP2/0 cells were combined with splenocytes at a 1:7 ratio. The positive hybridomas were screened by ELISA. The hybridomas were cloned by limiting dilution and the produced immunoglobulins were purified on Protein A Sepharose CL-4B column as described [19].

Polyclonal antibody production by cDNA immunization

The polyclonal antibodies against human NTPDase2 were raised in rabbit. Samples of 700 μ g of complementary DNA (cDNA) encoding for human NTPDase2 diluted in 1 mL of phosphate-buffered saline (PBS) 0.8 $\times$ were injected at 10 intradermic sites of 50 μ L each and two intramuscular sites of 250 μ L at each of the five injections at days 0, 14, 28, 98, and 169. When the titer was not sufficiently high after the third injection, as tested by western blot, the fourth injection was

followed by an additional final injection before bleeding the animal. Note that the antibodies to NTPDases as well as to nucleotide pyrophosphatase/phosphatases (NPPs) and ecto-5'-nucleotidase (CD73) that we have generated are available at <http://ectonucleotidases-ab.com/>.

Cell transfection and western blot

African green monkey kidney (COS-7) cells and HEK 293T cells were cultured and transiently transfected as indicated with human NTPDase1, -2, -3, or -8 cDNA constructs, mouse NTPDase2 or rat NTPDase2 as described previously [2]. For western blot assays, lysates from transfected or non-transfected COS-7 cells (6 µg, unless otherwise indicated) were resuspended in NuPAGE sample buffer, separated on NuPAGE 4–12% Bis-Tris gels under reduced or non-reduced conditions, as indicated, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) by electroblotting according to the manufacturer's recommendation. Membranes were then blocked with 2.5% non-fat milk in PBS containing 0.15% Tween@20 (pH 7.4) O/N at 4 °C and subsequently probed with the primary antibodies for 1.5 h at room temperature (RT). The membrane was then incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies for 1 h at RT and then developed with the Western Lightning™ Plus-ECL system (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). In some experiments, a gel with a 6-cm well containing 120 µg of lysates, transferred and blocked as described above, was probed with the antibodies using the Mini-Protein II multiscreen apparatus (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) in which 20 antibodies can be tested simultaneously with one gel.

ELISA and isotyping

ELISA was performed on COS-7 cells transiently transfected with human NTPDase2 or non-transfected as detailed here. One day after transfection, the cells were trypsinized and plated in 96-well plates (20,000 cells per well) to allow 80% confluence the following day. The next day, the cells were washed with PBS 0.05%, Tween 20 (PBS-T) and fixed with acetone-10% formalin neutral buffered (Fisher Scientific, Ottawa, ON, Canada) at a ratio of 19:1. Washed cells were incubated for 1 h at 37 °C in the following blocking solution: 0.5% bovine serum albumin diluted in PBS-T. After washing, the supernatant from each hybridoma was added to a well and incubated for 2 h at RT, followed by four washing steps. Then, a goat anti-mouse IgG conjugated to HRP (1:2500) diluted in the blocking solution was incubated for 2 h at RT followed by four washing steps. The Enhanced K-Blue® Substrate (Neogen Corporation, Lansing, MI, USA) was then added for 15 min and the reaction was stopped by the addition of

an equal volume of 2 N sulfuric acid. The absorbance at 450 nm was recorded.

The isotypes of the antibodies produced by the hybridomas were determined by a Mouse Immunoglobulin Isotyping ELISA Kit (BD Bioscience, Mississauga, ON, Canada) according to the manufacturer's instruction. In brief, monoclonal rat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM were coated O/N in 96-well plates. After a washing step and a blocking treatment, each monoclonal anti-human NTPDase2 antibody was transferred to the wells. After washing steps, rat anti-mouse Igs conjugated to HRP was added to each well, and revealed with a substrate provided in the kit. The plate was then read at 450 nm.

Immunocytochemistry and immunohistochemistry on frozen section

Human intestine cryosections or COS-7 cells previously transfected or not, were detached from the plates 24 h after transfection with a citric saline solution (135 mM potassium chloride, 15 mM sodium citrate) and placed on a microscope slide using cytocentrifuge and fixed with cold acetone and 10% neutral buffered formalin (Fisher Scientific, Ottawa, ON, Canada) (19:1). Samples were then blocked for 30 min in a PBS solution containing 5% bovine serum albumin or 7% normal goat serum for immunohistochemistry and immunocytochemistry, respectively. Tissue sections or COS-7 cells were incubated with the indicated primary antibody at 4 °C followed by a 10-min treatment with 0.3% H₂O₂ in PBS to inactivate endogenous peroxidase and then an avidin/biotin solution (Avidin/Biotin Blocking kit; Vector Laboratories) to prevent nonspecific staining due to endogenous avidin or biotin. This step was followed by incubation with an appropriate biotin-conjugated secondary antibody at a dilution of 1:1000. The avidin-biotinylated HRP complex (VectaStain Elite ABC kit; Vector Laboratories) was added to optimize the reaction. Peroxidase activity was revealed with DAB as the substrate. Nuclei were counterstained with aqueous hematoxylin (Biomedex, Foster City, CA, USA) in accordance with the manufacturer's instructions.

Immunohistochemistry on paraffin section

Human intestine paraffin sections were deparaffinized in toluene and rehydrated in a graded ethanol series (100, 90, 70, and 0% in distilled water). Slides processed for polyclonal antibodies were then incubated with sodium citrate buffer while slides processed for mouse monoclonal anti-human NTPDase2 were incubated with Tris-EDTA buffer (TE). Antigen retrieval with sodium citrate consisted of incubating slides in 10 mM sodium citrate with 0.05% Tween 20 at 95 °C for 5 min (pH 6.0) and then allowing the slides to cool to room temperature. Antigen retrieval with TE buffer consisted of incubating slides in 1 mM

EDTA with 0.5% Triton X-100 and 50 mM Tris at 37 °C for 7 min (pH 8.0). All slides were blocked with 5% bovine serum albumin after antigen retrieval and then processed for immunohistochemistry as described above.

Whole-mount preparation and immunohistochemical labeling

Samples of live, full-thickness jejunum were collected and placed in chilled Dulbecco's modified Eagle Medium (DMEM)/F-12 medium during transportation to the laboratory. Tissue was preserved in Zamboni's fixative before dissecting. Longitudinal muscle myenteric plexus (LMMP) whole-mount preparations were obtained by microdissecting the mucosa, submucosa, and circular muscle layers. LMMP preparations underwent three 30-min washes in 0.1% Triton X-100 phosphate-buffered saline (PBS) followed by a 3-h incubation in blocking solution containing 4% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin. Preparations were sequentially incubated with primary antibodies for 48 h at 4 °C followed by secondary antibodies for 24 h at 4 °C before mounting. Antibody specificity for commercial antibodies against SOX10 (Abcam #ab27655, 1:1000 dilution) and Hu C/D (Invitrogen #A21272, 1:200 dilution) was confirmed by preadsorption with the corresponding control peptides. Specificity was controlled for antibodies synthesized in-house with a mouse IgG2b monoclonal isotype control for the mouse NTPDase2 monoclonal antibodies or with preimmune serum for the rabbit polyclonal antibodies. Fluorescent labeling was visualized by confocal imaging through the Plan-Apochromat 60× oil immersion objective (1.42 numerical aperture) of an inverted Olympus Fluoview FV1000 microscope (Olympus, Center Valley, PA).

Flow cytometry

HEK 293T cells non-transfected or transfected with the indicated plasmid, were detached from the plates with a citric saline solution (135 mM potassium chloride, 15 mM sodium citrate). Samples of 2.5×10^5 cells per tube were washed with an ice-cold PBS solution containing 1% FBS (FACS buffer) followed by a 30-min incubation with the primary antibodies in FACS buffer on ice. The primary antibodies were either purified mouse monoclonal antibodies or rabbit polyclonal antibodies to human NTPDase2, or, as a negative control, mouse IgG2b irrelevant antibodies (Sigma-Aldrich) or rabbit preimmune sera, respectively. After washes with FACS buffer solution, the cells were incubated with an appropriate Alexa-conjugated secondary antibody for 30 min on ice, washed with FACS buffer, and analyzed by flow cytometry (BD LSR II, BD Biosciences, San Jose, CA USA).

ATPase activity assays

Enzymatic assays were performed as previously described [19] using cell lysates from human NTPDase2 transfected COS-7 cells at 37 °C in three different buffers: a modified Ringer buffer (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 , 5 mM dextrose, 80 mM Tris-HCl, pH 7.4); a Tris/calcium (Tris-Ca) buffer (5 mM CaCl_2 , 80 mM Tris-HCl, pH 7.4); or a Tris/magnesium (Tris-Mg) buffer (5 mM MgCl_2 , 80 mM Tris-HCl, pH 7.4). After a 20-min preincubation of the enzyme (cell lysate) with the purified monoclonal antibodies (0.05–10,000 ng/mL), the reaction was started with the addition of the substrate (ATP, 100 μM), and stopped 8–10 min later by the addition of the malachite green reagent which contains 4.72 N H_2SO_4 . The inorganic phosphate released during the enzymatic reaction was measured as previously described with the malachite green assay [20].

Inhibition by hN2-D5_s on intact COS-7 cells transfected with human NTPDase2 expression vectors, was measured in 24-well plates in Ringer buffer after 10 min preincubation with hN2-D5_s, or an irrelevant IgG2b as a negative control, followed by the addition of 100 μM ATP. To stop the reaction, 200 μL of cell supernatant was transferred to a tube containing 50 μL of malachite green reagent. The activity obtained on non-transfected cells was subtracted from the activity obtained with transfected cells for each corresponding condition.

Results

Production and characterization of monoclonal antibodies to human NTPDase2

Our previous attempts to produce monoclonal antibodies using cDNA immunization were unsuccessful. Therefore, we tried to increase the titer of the antibodies by making a final boost with HEK 293T cells expressing human NTPDase2 proteins by transient transfection. This procedure was carried out with the following mouse for which the serum collected 12 days after the third injection had the highest titer, as determined by western blot in non-reducing conditions. B cells collected from the spleen were fused with myelomas cells and the supernatants of the unpurified hybridomas were tested by ELISA in 96-well plates. The hybridomas that produced a positive signal on COS-7 cells transfected with human NTPDase2 and a negative signal on non-transfected COS-7 cells (Fig. 1) were recloned by successive steps of serial dilution to ensure that the selected clones were pure. Figure 1 shows the data obtained for the supernatants of eight hybridomas out of approximately 1000 tested; including wells where hybridomas did not grow. Thirty hybridomas were identified by this ELISA technique and the four with the

Fig. 1 Identification of hybridomas that produce anti-NTPDase2 antibodies. Supernatants of hybridomas were tested by ELISA on COS-7 cells transfected (filled bars), or not (open bars), with human NTPDase2 plasmid and absorbance was measured at 450 nm. The group on the left shows some representative data obtained with supernatants collected from hybridomas after the first screening (unpurified clones) and the group of data on the right shows the measurements obtained on the clones after recloning each positive hybridomas. The hybridomas producing an antibody that detects human NTPDase2 were identified as clone B2_s, D5_s, H9_s, and H10_s

highest signals on ELISA were further evaluated, namely hN2-B2_s, hN2-D5_s, hN2-H9_s, and hN2-H10_s (Fig. 1). We performed an ELISA assay to determine the isotype of these monoclonal antibodies and found that all four hybridomas produced IgG2b (data not shown).

The four monoclonal antibodies to human NTPDase2 were then tested by western blot using lysates from COS-7 cells transfected or not with plasmid-encoding human NTPDase2. As shown in Fig. 2, the four monoclonal antibodies specifically recognized human NTPDase2 only in non-reducing conditions. Indeed, no signal was detected in the presence of β -mercaptoethanol (Fig. 2b).

The potential cross-reactivity of these monoclonal antibodies with other NTPDases was then tested on lysates from COS-7 cells transfected with plasmids expressing each of the other closely related E-NTPDase members that also bind the plasma membrane (i.e., NTPDase1, -3, and -8) as well as with plasmids expressing mouse and rat NTPDase2. As shown in Fig. 2c, hN2-D5_s did not give any reaction with any of these NTPDases. Similar results were obtained for the three other monoclonals (data not shown).

We then tested whether these antibodies could detect the native NTPDase2 expressed at the cell surface. The four monoclonal antibodies, hN2-B2_s, hN2-D5_s, hN2-H9_s, and hN2-H10_s gave a similar pattern of staining by immunocytochemistry on human NTPDase2-transfected COS-7 cells (Fig. 2d). No signal could be detected on non-transfected COS-7 for any of the four monoclonals. This control is shown on Fig. 2d for hN2-D5_s. Further experiments by flow cytometry gave similar results (Fig. 3). As for western blot, these

antibodies appear specific for human NTPDase2 as no shift was detected on HEK 293T cells transfected with either mouse or rat NTPDase2 as shown in the lower panels of Fig. 3 for hN2-H9_s. Similar results were obtained with the other three monoclonal antibodies (data not shown).

Interestingly, one of these monoclonal antibodies reduced the ATPase activity of human NTPDase2 by half (Fig. 4a). The inhibitory effect of this clone (hN2-D5_s) was tested in Ringer buffer, in Tris/calcium buffer and in Tris/magnesium buffer. A similar inhibition pattern was obtained in the Ringer and in the Tris-Ca buffer. The inhibition of human NTPDase2 in the presence of hN2-D5_s was slightly lower in the Tris-Mg buffer (Fig. 4b). Ringer buffer was then selected for the following assays. All activity tests were performed on protein extract of at least two different preparations for each enzyme. Although the specific activity varied slightly from batch to batch, the percentage of inhibition was stable in the presence of hN2-D5_s or its isotype control. As for western blot and flow cytometry experiments, the inhibition in the presence of hN2-D5_s was also specific to human NTPDase2 proteins. Indeed, no inhibition was detected when hN2-D5_s was incubated with cell lysate from the other human NTPDases (NTPDase1, -3, and -8) or from other species of NTPDase2 (mouse and rat; Fig. 4c). The clone hN2-D5_s also had the ability to block the activity of human NTPDase2 located at the cell surface (Fig. 4d).

Production and characterization of polyclonal antibodies to human NTPDase2

Polyclonal antibodies to human NTPDase2 were produced by cDNA immunization in three rabbits named hN2-1_L, hN2-2_L, and hN2-3_L. Sera were collected after the fourth (I₄) and the fifth injection (I₅), aliquoted and stored at -80°C with 10% glycerol. The specificity of these antibodies was tested by the techniques detailed below.

Polyclonal rabbit sera to human NTPDase2 were first tested by western blot in non-reducing conditions using protein extracts from transfected cells. A strong positive reaction was obtained with the immune serum hN2-3_LI₅ (Fig. 5a) as well as for hN2-2_LI₅ (data not shown). A weaker signal was obtained for the hN2-1_LI₅ serum (data not shown). As for the monoclonal antibodies, the polyclonal antibodies did not recognize human NTPDase2 protein in reduced conditions (Fig. 5a for hN2-3_LI₅). The sera of these three rabbits were then tested by immunocytochemistry on non-transfected COS-7 cells or transfected with a plasmid expressing human NTPDase2. Our immunocytochemistry data (Fig. 5b) indicated that hN2-3_LI₅ reacts with COS-7 cells transfected with human NTPDase2. Similar results were obtained for hN2-2_LI₅ and a weaker reaction with hN2-1_LI₅ (data not shown). The antibody hN2-3_LI₅ was also able to bind the native form of human NTPDase2 as seen by flow cytometry in Fig. 5c. No signal was obtained when non-transfected cells were incubated with

Fig. 2 Specificity of the mouse monoclonal antibodies to human NTPDase2 by western blot and immunocytochemistry. **a** Western blot on lysates (120 μ g, for one large well) from COS-7 cells transfected with a plasmid-encoding human NTPDase2 in non-reducing conditions with the anti-human NTPDase2 monoclonal antibodies produced by each one of the four hybridomas: hN2-H10_s, hN2-D5_s, hN2-B2_s, and hN2-H9_s. **b** Western blot on lysates from COS-7 cells transfected with a plasmid-encoding human NTPDase2 in reduced condition and incubated with the anti-human NTPDase2 antibodies hN2-H9_s, hN2-D5_s, hN2-H10_s, and hN2-B2_s (left panel). A positive control was performed on the same gel using a human NTPDase2 COS-7 lysate in non-reducing condition and incubated with hN2-D5 antibody (right panel). An empty lane was put in between to avoid the

diffusion of mercaptoethanol in the control sample. **c** Specificity of the hN2-D5_s antibody (0.25 μ g/mL) by western blot on lysates from non-transfected COS-7 cells (COS) or transfected with a plasmid expressing either human NTPDase1 (hN1), -2 (hN2), -3 (hN3), -8 (hN8), mouse NTPDase2 (mN2), or rat NTPDase2 (rN2) in non-reducing condition. **d** Immunocytochemistry on COS-7 cells transfected with human NTPDase2 or on non-transfected COS-7 (third upper panel) probed with the mouse monoclonal anti-human NTPDase2 antibodies hN2-B2_s, hN2-D5_s, hN2-H9_s, and hN2-H10_s, or with the irrelevant IgG2b as a negative control. hN2-D5_s was also probed on non-transfected COS-7 as an additional negative control. All antibodies were tested at 0.5 μ g/mL. Scale bar represents 25 μ m

the immune serum. A similar result was obtained with the hN2-2_LI₅ antiserum and a weaker positive signal was observed with hN2-1_LI₅ (data not shown).

Table 1 summarizes the characteristics of the antibodies that we have generated here against human NTPDase2.

NTPDase2 localization in human intestine

These monoclonal and polyclonal antibodies were tested by immunohistochemistry on human intestines to define the cells

that express NTPDase2. Figure 6 shows that the monoclonal anti-human NTPDase2 hN2-H9_s and the rabbit anti-human NTPDase2 antiserum hN2-2_LI₅ detected the presence of NTPDase2 protein in the nerve structures of human colon on both cryosections (Fig. 6a) and paraffin sections (Fig. 6b), although with a weaker reaction in the latter. In cryosection, similar results were obtained with the three other hybridomas and with the two other rabbit sera (data not shown).

In paraffin sections, the most intense reaction was obtained with hN2-H9_s followed by hN2-D5_s and hN2-B2_s. No signal

Fig. 3 Specificity of the mouse monoclonal antibodies to human NTPDase2 by flow cytometry. Flow cytometry on non-transfected HEK 293T cells (293 non tf) or transfected with human, mouse or rat

NTPDase2 cells incubated with hN2-D5_s, hN2-B2_s, hN2-H10_s, and hN2-H9_s or their isotype control (IgG2b) at the concentration of 1 µg/mL

was obtained with the other monoclonal antibody (hN2-H10_s) in these conditions. Other buffers to unmask antigens have been tested for mouse anti-human NTPDase2 monoclonal antibodies without success. This includes sodium citrate buffer (as described in the “Materials and methods” section) incubated for 5 and 20 min as well as proteinase K (20 µg/mL) diluted in TE buffer applied on the sections for 7 min at 37 °C, (as described in the “Materials and methods” section). For the rabbit antibodies, similar results were obtained for the three sera. See Fig. 6b for hN2-2_LI₅ and data not shown for the other two antisera. No other antigen retrieval technique was tested for the rabbit polyclonal antibodies.

To determine whether NTPDase2 is present in neuronal cells or in surrounding glial cells, colocalization experiments were performed using whole mounts of the myenteric plexus from human jejunum using the glial cell marker SOX10 and the neuronal marker Hu C/D. The anti SOX10 antibody produced in rabbit was used in double staining with the anti-NTPDase2 monoclonal antibodies and the Hu C/D antibody, which is from mouse, was tested with the rabbit NTPDase2 antisera. The results presented in Fig. 6c suggest that NTPDase2 is expressed by glial cells surrounding nerve structures in the human jejunum.

Discussion

In the present study, we generated four hybridomas that produce IgG2b monoclonal antibodies (from which at least two are different) as well as three polyclonal rabbit antisera that are specific to human NTPDase2. These antibodies are compatible with all techniques tested including western blot, flow cytometry, immunocytochemistry, and immunohistochemistry. The antibodies are extremely specific and did not bind to other plasma membrane bound-related NTPDases (human NTPDase1, -3, -8) nor to the other NTPDase2 species tested (mouse and rat).

For most techniques, the monoclonal antibodies gave a stronger signal over background to human NTPDase2 than the polyclonal antisera, especially on western blot (Table 1). The polyclonal antibody that gave the best reaction over background was hN2-3_LI₅ in western blot, immunocytochemistry, and flow cytometry and hN2-2_LI₅ in immunohistochemistry. The best monoclonal was hN2-H9_s but the other three monoclonal antibodies also produced similar staining for all techniques. Indeed, all data generated with hN2-H9_s consistently gave a better signal over background when compared with the other three monoclonals. The above qualitative assessment is

Fig. 4 hN2-D5_s specifically inhibits human NTPDase2. **a** Each monoclonal anti-human NTPDase2 antibody was incubated with protein extracts from COS-7 cells expressing human NTPDase2. The ATPase activity obtained with the protein extract alone was set as 100% which was 430 ± 190 nmol Pi/mg protein/min. **b** ATPase inhibition titration of hN2-D5_s in three buffers: Tris-Ca, Tris-Mg, and Ringer buffers as described in experimental procedures. The results are expressed in percentage of activity of human NTPDase2 in presence of the monoclonal antibody compared to the activity obtained in the absence of antibody which was 393 ± 104 , 1110 ± 224 , and 363 ± 88 nmol Pi/mg protein/min for the Ringer, the Tris-Ca, and the Tris-Mg buffers, respectively. **c** Specificity of the inhibition of hN2-D5_s on human NTPDase1 (hN1), -2 (hN2), -3

(hN3), and -8 (hN8), and mouse NTPDase2 (mN2) and rat NTPDase2 (rN2). The 100% ATPase activity for human NTPDase1, -2, -3, -8, mouse and rat NTPDase2 was 446 ± 75 , 361 ± 120 , 202 ± 117 , 207 ± 29 , 990 ± 639 , and 468 ± 148 nmol Pi/mg protein/min, respectively. **d** Influence of hN2-D5_s on the NTPDase2 ATPase activity expressed at the cell surface of COS-7 cells transfected with human NTPDase2. The ATPase activity obtained on intact transfected cells without monoclonal antibody was set as 100% and was 1084 ± 48 nmol Pi/min/well. Data are presented as the mean $\pm$ SEM of three experiments carried out in triplicate (**a–c**) and in duplicate (**d**). ** $p < 0.01$, *** $p < 0.001$ (1-way ANOVA (**a**) and 2-way ANOVA for (**c**, **d**))

confirmed quantitatively by the data generated by flow cytometry as seen on Fig. 3.

Importantly, hN2-D5_s specifically inhibited human NTPDase2. Therefore, there are at least two different monoclonal antibodies. Indeed, it is possible that the other three monoclonals (hN2-B2_s, hN2-H9_s, and hN2-H10_s) derive from the same original B cell clone because they are all of the IgG2b subtype and all gave similar results in each assay used here. Note that the signals on paraffin sections were weak with the monoclonal antibodies and we therefore prefer not to consider the minor differences observed with this technique to discriminate with certitude whether some other clones are different even if this technique suggests that hN2-H9_s is a different monoclonal antibody. Another characteristic of interest for some sandwich assays is whether these antibodies recognize distinct and non-overlapping epitopes. This characteristic is left for future studies if needed.

The fact that all monoclonal and polyclonal antibodies detected NTPDase2 only in non-reduced condition suggests that the most antigenic epitope(s) generally recognized is(are) part of a tertiary structure of the protein that involves disulfide bridges. This is a common feature that we have observed for most antibodies generated against native forms of the plasma membrane bound NTPDases.

The cDNA immunization technique that we used here was also useful for the production of antibodies against several other ectonucleotidases [16, 18, 33–36] (ectonucleotidases-ab.com) as well as against rat and mouse NTPDase2 [7, 8]. These antibodies allowed for the identification of the cells that express NTPDase2 in various tissues. This information formed the basis of many current hypotheses regarding NTPDase2 expression and function as discussed in the introduction. In the present work, we used our antibodies to show that enteric glial cells in the human intestine express NTPDase2. This is in agreement with our prior observations

Fig. 5 Specificity of the rabbit polyclonal antiserum hN2-3L5 to human NTPDase2 by western blot, immunocytochemistry and flow cytometry. **a** Western blot on lysates (120 µg for one large well) from COS-7 cells transfected with a plasmid encoding human NTPDase2 in non-reducing conditions (N.R.) (left panel) or in reduced condition (red) (right panel) incubated with the rabbit polyclonal anti-human NTPDase2 antibody hN2-3L5 or its preimmune serum (both 1:500). **b**

Immunocytochemistry on non-transfected COS-7 cells (left panels) or transfected with human NTPDase2 encoding plasmid (right panels) and probed with hN2-3L5 (upper panels) or with its preimmune serum (lower panels) (both 1:500). Scale bar represents 25 µm. **c** Flow cytometry on non-transfected HEK 293T cells (293 non tf) or transfected with human NTPDase2 cells incubated with hN2-3L5 (upper panel) or its preimmune serum (lower panel) (both 1:100)

in the myenteric plexus of rat [21], mouse, and guinea pig [22] colons. The consistency of this observation across different species suggests a general role of NTPDase2 in the neural regulation of intestinal functions in mammals.

One of the key findings of our study is that one of the monoclonal antibodies specifically inhibits the enzymatic activity of human NTPDase2. Some molecules have already been reported to inhibit NTPDases such as ARL 67156 [23,

24], PSB 069 [25], and sodium azide [26, 27] but they do not discriminate between NTPDases. Other compounds such as reactive blue-2, suramin, and derivatives also inhibit NTPDases but none of them are selective and their use is complicated by the fact that they also act as antagonists for several P2 receptors. Some polyoxometalates have been described that act as NTPDase's inhibitors [28] and one of these compounds is reported to be specific for human NTPDase1

Table 1 Summary of the characteristics of the human NTPDase2 antibodies

Antibody	Applications						
	WB		IHC		ICC	FACS	ATPase inhibition
	N.R.	Red.	Frozen section	Paraffin section			
Mouse monoclonal antibodies							
hN2-B2 _s	++	—	++	+	++	++	—
hN2-D5 _s	++	—	++	+	++	++	++
hN2-H9 _s	++	—	++	+	++	++	—
hN2-H10 _s	++	—	++	—	++	++	—
Rabbit polyclonal antibodies							
hN2-1 _L I ₅	+	—	++	+	+	++	N.T.
hN2-2 _L I ₅	+	—	++	+	++	++	N.T.
hN2-3 _L I ₅	+	—	++	+	++	++	N.T.

Results “—” absence of reaction, “+” weak positive reaction, “++” strong and specific reaction and therefore antibody appropriate for research purposes

WB western blot, N.R. non-reduced condition, Red reduced condition, IHC immunohistochemistry, ICC immunocytochemistry, FACS fluorescence-activated cell sorting, N.T. not tested

Fig. 6 Immunolocalization of NTPDase2 in human intestine. Localization of human NTPDase2 in cryosection (a) or paraffin (b) human colon or within the myenteric plexus of human jejunum (c) using the monoclonal antibody hN2-H9_s (upper panels) or the polyclonal antibody hN2-2L_{I₅} (lower panels). An IgG2b irrelevant

antibody and a preimmune serum (hN2-2L_{Pi}) were used as negative controls. c Glial cell marker, SOX10, and the neuronal marker, Hu C/D, was used for colocalization. Counterstaining of nuclei (blue) was performed with aqueous hematoxylin (a). Scale bar represents 20 μm (a, b) and 100 μm (c)

(PSB-POM142) [29]. Another, PSB 06126, is selective for rat NTPDase3 [25]. Recently, two inhibitors of NTPDase2 have been developed [30, 31], but until now, no blocking monoclonal antibodies have been reported to inhibit human NTPDase2.

Our blocking antibody has some obvious applications for basic research studies on human NTPDase2 but it might also be of interest in clinical applications. For example, a recent study showed that the implantation of C6 rat glioma cells overexpressing NTPDase2 produced larger tumors [32]. These data suggest that NTPDase2 could be a potential therapeutic target for treating some cancers and our blocking antibody might be an interesting tool.

In summary, we have generated three rabbit antisera and four hybridomas (from which at least two are distinct) that produce IgG2b antibodies to human NTPDase2. The

generated antibodies in this study are specific and efficient at least for western blot, for flow cytometry, for immunocytochemistry, and for immunohistochemistry in non-reduced conditions. These antibodies are highly specific to human NTPDase2 as they did not cross-react with any of the other related NTPDases. In addition, one hybridoma, hN2-D5_s produced an antibody that specifically inhibits human NTPDase2. In conclusion, we have generated powerful tools to study human NTPDase2 that are made available at <http://ectonucleotidases-ab.com/>.

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Compliance with ethical standards

Conflict of interest Julie Pelletier declares that she has no conflict of interest.

Hervé Agonsanou declares that he has no conflict of interest.

Ninotchka Delvalle declares that she has no conflict of interest.

Michel Fausther declares that he has no conflict of interest.

Mabrouka Salem declares that he has no conflict of interest.

Brian Gulbransen declares that he has no conflict of interest.

Jean Sévigny declares that he has no conflict of interest.

JS declares no personal conflict of interest.

The amount requested for these reagents are used to pay for the cost of production and any excess is reinvested to generate and characterize new antibodies.

Ethical approval All procedures were approved by the Canadian Council on Animal Care and the Université Laval Animal Welfare Committee. Human intestines were obtained under approved institutional review board protocol (Comité Éthique de la Recherche du CHU de Québec - Université Laval) following written consent. Work involving human jejunum whole preparation was approved by the institutional review board of Michigan State University (IRB 13-945M).

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