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Inducible Nitric Oxide synthase in duodenum of children with *Giardia lamblia* infection

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Abstract: The investigation were performed on children with *Giardia lamblia* infection, diagnosed on the basis of positive stool tests for Giardia antigen (Elisa) or by microscopical detection of trophozoites in duodenal fluid. In duodenal biopsies morphological studies and immunohistochemical reaction for inducible nitric oxide synthase (iNOS) were performed. The control group was made up of duodenal tissue of children with excluded giardiasis and inflammation of the upper part of gastrointestinal tract. The duodenal biopsies from children without *Giardia lamblia* infection were found to have a high immunoreactivity for iNOS in enterocytes, the cells of intestinal crypts, endothelial cells of vessels and connective tissue cells of lamina propria. In children with giardiasis: in some biopsies the expression of iNOS was as high as in control group, in others was weaker detectable and the shortening of intestinal villi was seen. There were also duodenal biopsies with the lack of immunoreactivity for iNOS, with shorter villi and a large amount of mucus in the intestinal epithelium. Beside of goblet cells, also enterocytes were loaded with mucus. The pathological changes may cause malabsorption and also may have a negative influence on the defense of the intestinal wall against *Giardia lamblia* infection. The different morphological and immunohistochemical results in the duodenum of children with giardiasis can elucidate a variety of clinical symptoms from asymptomatic to severe infection.

Key words: duodenal biopsies, *Giardia lamblia*, iNOS expression

Introduction

Protozoan *Giardia lamblia* is a widespread parasite of the gastrointestinal tract in human as well as domestic and wild animals [1,2]. It mainly inhabits the small intestine but can also live in the gallbladder and pancreatic tracts [3]. The parasite exist in two forms: the infectious endospore cysts, which are resistant to many environmental factors and trophozoites causing clinical symptoms and the disease [4]. Excytation of ingested cysts is due to the low pH level in the stomach as well as elevated pH and proteolytic enzymes in duodenum, whereas encytation requires elevated pH and bile [3]. Trophozoites are able to attach to the microvillous brush border of enterocytes [5] and penetrate crypts but do not invade mucosa [3]. However, there are also investigations indicating that some strains of *Giardia lamblia* disrupt the tight-junctional ZO-1 between intestinal epithelial cells and significantly increase paracellular permeability due to Giardia induced enterocyte apoptosis [6-8]. The interaction between parasites and host shows a number of clinical symptoms. In many individuals, the infection remains asymptomatic whereas other patients exhibit severe symptoms [1,9]. The most important clinical signs of giardiasis include: loss of apetite, abdominal pain, chronic diarrhea, nausea, vomiting and malabsorption. The duration period of disease can vary considerably. In majority of cases giardiasis is a self-limiting process, indicating existence of a host defense against the parasite. One of them is nitric oxide (NO) produced by nitric oxide synthase (NOS). In the digestive tract NO performs many functions including: peristaltic movement, action of sphincters, enlargement of

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the mucosal blood vessels, inhibition of plateletes and leukocyte adhesion and/or aggregation within the vasculature [10-12]. NO is also involved in the host defense against invading bacteria and parasites.

Nitric oxide – a short-living free radical with biological function, is synthesized from L-arginine through the activation of nitric oxide synthase (NOS). The enzyme exist in three isoforms: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS). Two of them eNOS and nNOS are constitutive calcium-dependent enzymes (cNOS) [11]. Apart from vascular endothelial cells and neuronal cells they are also expressed in other type of cells [13-15]. The third isoform – inducible nitric oxide synthase (iNOS) is calcium-independent and after activation by cytokines and bacterial endotoxins, produces a large amount of NO for an extended period [12,16]. In our former studies we showed iNOS mRNA in a freshly isolated epididymal epithelial cells of control rats [13] and we also showed iNOS immunoexpression in the cells of testis and epididymis of rats, without induction with endotoxins and cytokines [17]. The recent findings also revealed the constitutive expression of iNOS in horse testis interstitial cells and in epididymal epithelial cells [14].

In polarized intestinal epithelial cells the stable end products of NO, nitrite and nitrate are detected at the apical side of the cells [18,19]. Because trophozoites remain in the close contact with the epithelial cells in duodenum [20], NO may be a potential host defense against *Giardia lamblia*.

Therefore the aim of the study was to investigate the expression of iNOS in duodenal bioptates from children with giardiasis.

Materials and methods

Patients. The study was performed on duodenal bioptates from children, aged 5 – 15 years, hospitalized in the Department of Pediatrics, Hematology and Oncology of Children, Pomeranian Medical University in Szczecin. The patients were admitted to hospital because of chronic abdominal pain and/or chronic diarrhea. Giardiasis was diagnosed in 16 children on the basis of positive stool tests for the Giardia antigen (Crypto/Giardia Duo Strip, Coris BioConcept, Belgium) or by microscopical detection of trophozoites in duodenal fluid, obtained by aspiration from a naso-duodenal tube.

In all children several tests were performed including routine serum and urine biochemical tests, stool culture, stool sample examination for Giardia antigen, ova of parasites and abdominal ultrasound. Gastroscopy with duodenal biopsy was also done to exclude oesophagitis, ulcer disease or celiac disease.

The control group was made up of duodenal bioptates of 8 children with excluded giardiasis and a negative result of gastroscopy.

Tissue specimens. The paraffin embedded tissue samples were processed and diagnosed by the Department of Pathology of Pomeranian Medical University. Routine staining with hematoxylin-eosin was performed. The spare samples were used for iNOS immunostaining and for mucins staining with PAS method according to Hotchkiss and McManus [21].

Immunohistochemistry. Paraffin-embedded sections (5 μm) of duodenal bioptates were immunostained for visualization of inducible nitric oxide synthase (iNOS). The immunohistochemistry (IHC) was performed using a specific primary antibody: rabbit, polyclonal anti-mouse inducible nitric oxide synthase (diluted 1:400) [SEROTEK Ltd, Kidlington, Oxford, UK; NOS-II; iNOS, AHP303, mouse macrophage NOS C-terminal peptide (1131-1144) + additional N-terminal Cys conjugated to KLM; recognize iNOS, and does not cross react with eNOS or nNOS; species cross reactivity: human & rat]. The deparaffinized sections were microwave irradiated in citrate buffer (pH 6.0) to heat induced epitope retrieval. After slow cooling to room temperature slides were washed in PBS twice for 5 min and then incubated for 60 minutes with primary anti-iNOS antibody. Next sections were stained with an avidin-biotin-peroxidase system with diaminobenzidine as the chromogen (EnVision$^+$System-HRP (DAB); Code K4010 DakoCytomation, Glostrup, Denmark) in conformity with staining procedure instruction included in Dako EnVision$^+$System. Sections were washed in distillated H_2O and counterstained with hematoxylin. For negative control, specimens were processed in the absence of primary antibody. Positive staining was defined microscopically by visual identification of brown pigmentation.

Ethical issues. Before a gastroscopy was performed an informed consent for all diagnostic and therapeutic procedures was obtained from parents or guardians of every single child. The study performed in this paper was retrospective based on biopsy specimens collected in years 2006-2008 and did not require additional endoscopies, biopsies or examinations.

Results

Histological examination of duodenal biopsies from 8 children without Giardia infection revealed no pathological changes in the mucosa layer. Therefore this group of 8 children was recognized as a control group. In slides obtained from these biopsies a strong iNOS-reactivity was detected in the basal and particularly in apical side of enterocytes in villi epithelium. Immunoexpression of iNOS was also noticed in the cells of intestinal crypts, in connective tissue cells and in the endothelial cells of lamina propria vessels (Fig. 1 A and B). In slides stained with PAS method, a thin layer of mucin was present on the surface of the intestinal epithelium. The goblet cells were filled with mucus (Fig. 2).

In children with *Giardia lamblia* infection the product of immunohistochemial reaction for iNOS was noticed in the cytoplasm of intestinal epithelial cells, with different intensity. In 5 biopsies the expression of iNOS was on the same level or nearly the same as in control group. In 8 cases the duodenal villi were shorter and iNOS was faintly detectable, especially in the epithelium of basal part of intestinal villi. There were also areas of lamina propria with negative reaction in intestinal crypts (Fig. 3). The amount and localization of mucin was similar in the above cases to that found in duodenal bioptates of children in control groups (Fig. 4).

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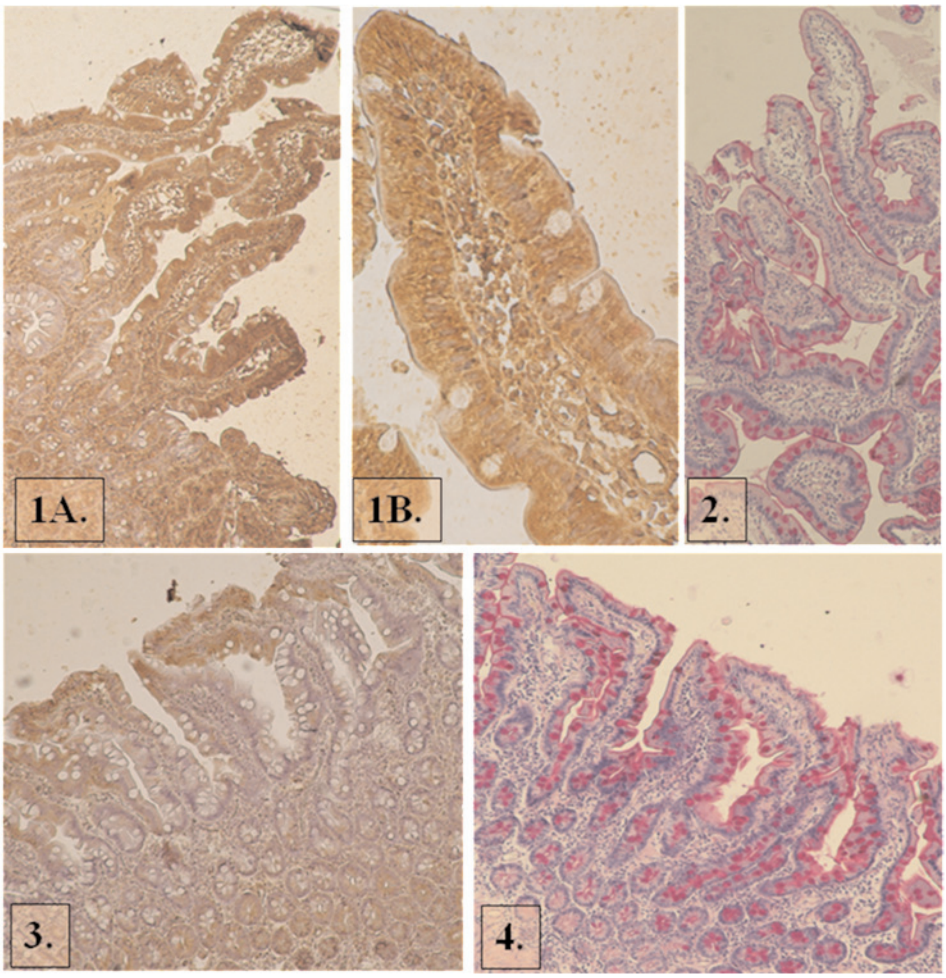

Fig. 1. Intensive immunohistochemical reaction for iNOS in duodenal epithelium, in cells of duodenal crypts, connective tissue cells and endothelial cells of vessels in bioptates of control group children (original magnification 1A ×160, 1B × 330). **Fig. 2.** Duodenal villi from bioptates of control group children. PAS-staining (original magnification ×160). **Fig. 3.** Immunohistochemical reaction for iNOS in *Giardia lamblia* infection. The villi are shorter and the immunoreactivity for iNOS in epithelium is limited to the upper part of intestinal villi. Some intestinal crypts are negative stained (original magnification ×160). **Fig. 4.** Lamina propria of duodenum in *Giardia lamblia* infection. PAS-staining (original magnification ×160).

In 3 Giardia-positive duodenal biopsies a notable degree of villi shortening was visible. In these biopsies the immunoexpression of iNOS both in enterocytes and intestinal crypts was negative (Fig. 5). A trace amount of immunoreactivity for iNOS was sometimes found in connective tissue cells, especially in upper part of villi (Fig. 7,8). In these cases there was a large amount of mucus in intestinal epithelium. Besides of goblet cells, mucus was also present in enterocytes (Fig. 6). Numerous lymphoidal cells were localized in lamina propia and epithelium.

Discussion

Intestinal epithelial cells are known to be NO-producing cells for affecting parasitic infection [22,23]. However NO generally shows a dual behavior: at physiological concentration, realized through the cNOS (calcium-depended NOS) regulates house-keeping functions, whereas under the influence of bacteria or parasites an overexpression of iNOS is observed [11]. The intestinal epithelial cells expressed iNOS in a large amount [1]. It has been also shown that the distal small bowel in mice expressed iNOS mRNA constitutively [2]. Also isolated human duodenocytes produce NO constitutively [24]. The intestinal epithelial cells dispose effective NO production, as a potential host defense mechanism against *Giardia lamblia*. Additionally, also fibroblasts and macrophages, cells immediately underlying the epithelium, can produce NO against Giardia, via diffusion through the epithelial layer.

In our study of children without giardial infection, the immunoreactivity for iNOS was intensive in enterocytes, largely confined to the apical side of the cells and in duodenal crypts. Immunoreactivity of iNOS was also confirmed in connective tissue cells and endothelial cells of lamina propria vessels. The immunoexpression for iNOS in normal enterocytes and other intestinal cells indicates, that the isoform of the enzyme is expressed in small intestine without inflammation or giardial infection. It should not be unexpected because the intestinal epithelium is always exposed to foreign antigens, including bacteria and their products as well as parasites. In this field our results are in agreement with other authors [22,23].

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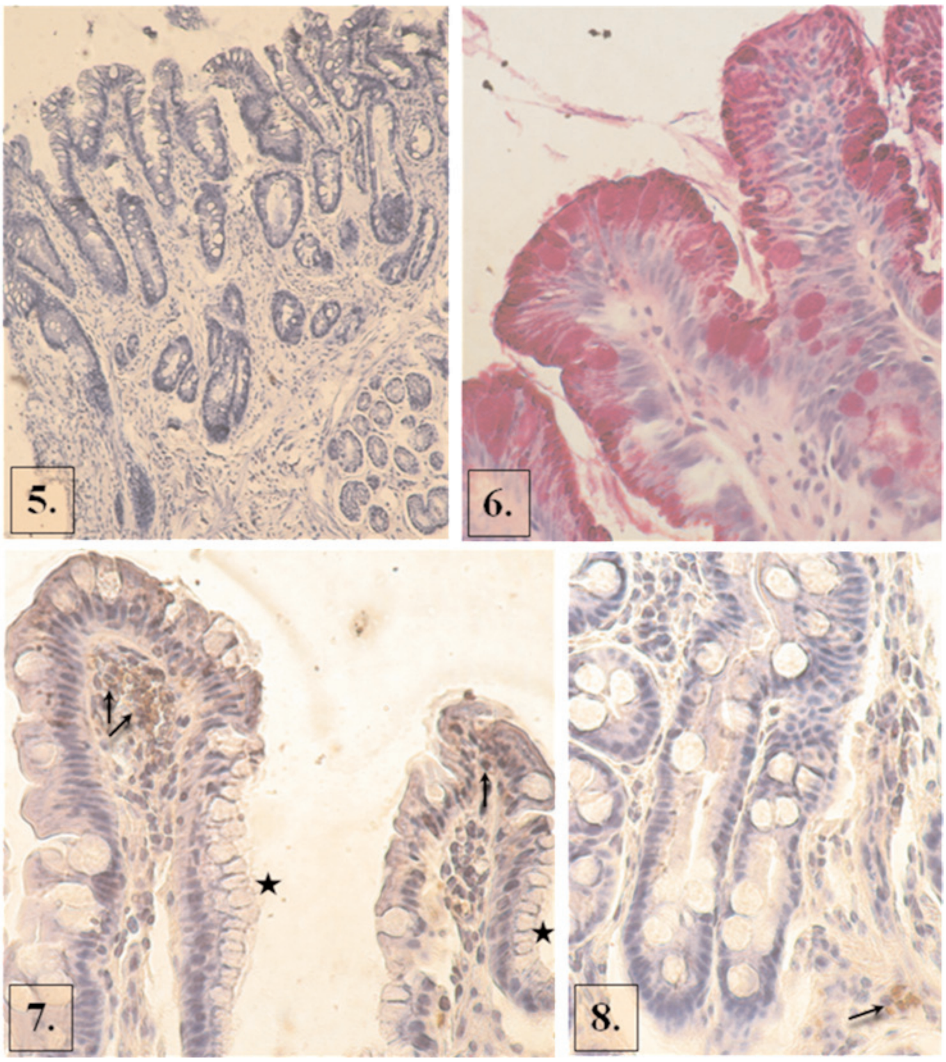

Fig. 5. Notable shortening of duodenal villi with lack of immunoexpression for iNOS in *Giardia lamblia* infection (original magnification ×160). **Fig. 6.** Large amount of mucus in enterocytes and goblet cells of the duodenal epithelium in *Giardia lamblia* infection. Numerous lymphoidal cells in the villi connective tissue. PAS-staining (original magnification ×330). **Fig. 7.** Duodenal villi in *Giardia lamblia* infection. The lack of immunohistochemical reaction for iNOS in the epithelium and the changed enterocytes (asterisks). Trace immunoreactivity for iNOS is present in some connective tissue cells (arrows) (original magnification ×330). **Fig. 8.** Duodenal crypts in *Giardia lamblia* infection. Negative reaction for iNOS. Only a few connective tissue cells reveal immunoreactivity for iNOS (arrow) (original magnification ×330).

Giardia lamblia was present in the duodenal fluid of 16 children. Among these in 5 biopsies there were no morphological alternations and the intensity of immunoreactivity for iNOS was on the similar level as in control bioptates.

Intestinal epithelial cells-derived NO, is a potential host defense against *Giardia lamblia*. As it was mentioned above, these cells revealed also increased expression of NOS in inducible isoform due to cytokines and microbial products and in constitutive one [1,2,24]. The stable end products of NO, nitrite and nitrate are preferentially detected mainly in the apical side of enterocytes [18,19]. It has been shown that NO has an inhibitory effect on growth and differentiation of *Giardia lamblia* without affecting viability. Therefore it has been found to be cytostatic but not cytotoxic for *Giardia lamblia* trophozoites [18]. Production NO together with other antigiardial host defense: antimicrobial peptides such as defensins and lactoferins [25-27], B cells [1,9], mast cells [28] and particularly secretory IgA antibodies [1,29] have an influence on duration of infection and severity of symptoms, leading in the majority of cases to self-limiting of the disease. This is probably the reason of lack of morphological alternations and the intensive immunoexpression for iNOS, the basic enzyme in synthesis of NO, in the biopsies of 5 children with *Giardia lamblia* infection.

However in bioptates from 8 children shortening of duodenal villi and weaker immunoreactivity for iNOS were detected, especially in the epithelium of basal part of intestinal villi. There were also areas of lamina propria with negative reaction in intestinal crypts. However in three Giardia-positive cases the duodenal villi were notable shorter and the immunoexpression of iNOS both in enterocytes and intestinal crypts was negative. A trace amount of immunoreactivity for iNOS was sometimes found in connective tissue cells, especially in upper part of villi. In these 3 cases a large amount of mucus in intestinal epithelium was found. Additionally, enterocytes similarly to goblet cells, also were loaded with mucus. Numerous lymphoidal cells

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were localized in the epithelium and in connective tissue villi.

The investigations performed *in vitro* in cultured human intestinal epithelial cells with *Giardia lamblia*, elucidated some mechanisms of NO production in the presence of the parasite in intestinum and the reaction between the pathogen and the host [1]. In spite of potential anti-giardial activity of NO, *Giardia lamblia* has strategies to evade this potential host defense. In co-cultures of human intestinal epithelial cells and *Giardia lamblia* trophozoites, *Giardia lamblia* inhibit the production of epithelial NO by taking up and effective consumption of arginine, the basic substrate in NO production. Arginine is an important source of energy for the parasite [1]. As a by-product of this reaction ornithine is released [30,31], which additionally competitively inhibit arginine uptake by the intestinal epithelium. Both mechanisms reduce the availability of arginine for enterocytes and in this way inhibit NO production. However this findings do not explain the lack of iNOS expression in the biopsies od 3 children with severe morphological alternations and high amount of mucus in the intestinal epithelium.

In the studies performed *in vitro* [18] *Giardia lamblia* infection had no effect on epithelial iNOS expression. It is difficult to say if the same situation takes place *in vivo*. In the normal human airways NO synthesis is due to the continuous expression of iNOS in the airway epithelial cells. However, removal of epithelial cells from the *in vivo* airway environment leads to rapid loss of iNOS expression, which suggests the expression to be depended upon specific condition and/or present factors [32]. It is therefore possible that epithelial iNOS expression can be regulated in different pattern, in this case probably due to appearance of mucus in enterocytes.

Intestinal mucins covering the surface of intestinal epithelium are large glycoproteins with high-charge density from sialic acid and sulfate residues as well as protease resistance and water holding capacity. Mucins synthetized by goblet cells can be categorized into two main classes based on their location and structure: membrane bound and secreted forms [33]. Only the latter contribute in the formation of mucus gel. Intestinal mucins are the constituent of luminal barrier function. This is the first line of host defense against enteric pathogens, preventing attachment of them to the mucosal surface. It has been shown that such mucus constituents as glucose and mannose [34], as well as N-acetyl-glucosamine, N-acetyl-galactosamine and fucose [35], inhibit the attachment of *Giardia lamblia* trophozoites to the epithelial cells. Langford *et al*. [29] demonstrated that antibodies of IgA are required for effective clearance of *Giardia muris* and *Giardia lamblia* from the murine host by immobilization or detachment of trophozoites from epithelium. IgA is the most abundant immunoglobulin in the mucosal secretion.

In the presence of pathogen in the intestinal lumen the host defence reacts quickly. In response to intestinal microbes mucin secretion is enhanced, and this rapid secretion is thought to provide an important mechanism of protection by eliminating intestinal pathogens. Many enteric microbes and their toxins are known to have a potent secretagogue effect on goblet cells [36]. This can lead to elimination of offending pathogen, because the epithelial surface sloughs off the tip of the villi every 72 hours, together with attached trophozoites.

In 3 Giardia-positive duodenal biopsies with large amount of mucus in the intestinal epithelium, the expression of iNOS was negative. The presence of mucus within the cytoplasm of enterocytes, in the same place where intensive reaction for iNOS was commonly confirmed, proves that the cells changed their function. This phenomenon besides of malabsorption may have a negative influence on the defence of the intestinal wall. From the medical history of these children it is known, that the 3 cases of giardiasis were resistant for treatment [unpublished data]. It is difficult to foresee if the stated changes are long-lasting or if they were for a short time. Nevertheless, the different morphological and immunohistochemical state of duodenal lamina propia can elucidate a variety of clinical symptoms from asymptomatic to severe infection in children with giardiasis.

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