EXPRESSION OF CYTOKINES AND MONOSACCHARIDE TRANSPORTERS IN THE DUODENAL MUCOSA OF PATIENTS WITH GASTROINTESTINAL SYMPTOMS IN RURAL THAILAND

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Abstract. Levels of cytokines and GLUT family monosaccharide transporters in the duodenal mucosa were examined in patients from Nong Khai, Thailand, who had underwent gastroscopy because of gastrointestinal problems. Duodenal biopsy specimens were collected from a total of 33 patients (24 males and 9 females, 45.0 ± 13.5 years old). Ten patients had present or recent intestinal helminth infections, including strongyloidiasis, taeniasis or ascariasis (group A), 7 were urease-test positive, indicating *Helicobacter pylori* infection (group B), and 16 had neither helminth infections nor urea-test positivity (group C). Total RNA was extracted from the biopsied specimens and a semi-quantitative RT-PCR was performed. The positivities for IL-13, IL-5 and IFN-γ mRNA expressions in the patients were 24.2, 60.6 and 100%, respectively, with the highest IL-13 and IL-5 positivities in group A, followed by group C and B. The IL-5 positive rate was significantly higher among patients with high peripheral blood eosinophil counts (>4%) than in patients with low peripheral blood eosinophil counts. GLUT-1 and GLUT-5 were detectable in all the patients. Although GLUT-1 expressions did not differ among groups A, B and C. GLUT-5 expressions were significantly lower in group B than in group C. These results indicate that helminth and *H.pylori* infections result in different immunopathological responses in the duodenal mucosa, lower expressions of type 2 cytokines and monosaccharide transporters in *H. pylori* infections than in helminth infections.

INTRODUCTION

Despite great strides in the improvement of public health, diarrheal diseases still remain a major health problem in rural Thailand. At Nong Khai Hospital, diarrhea was the most prevalent disease, 2.6-4.6% of all inpatients during 1999 to 2001 (Table 1). Infections with helminths and enteric pathogens may lead to a variety of conditions, including a loss of fluids, anorexia and malabsorption. Stool examinations of the general public of Nong Khai Province carried out during 1999 and 2001 revealed a 5.1-11.7% prevalence of hookworm, 4.9-9.0% prevalence of *Opisthorchis*, and a 19.2-23.6% prevalence of other parasites (Table 2). The high prevalence of food-borne and soil-transmitted helminth parasites in this region seem to reflect traditional agricultural and food handling practices, which may be an indicator of the high prevalence of diarrheal diseases caused by various enteric pathogens. Although intestinal helminthic parasites do not necessarily cause overt clinical signs, long-term infection induces malnutrition and results in retardation of the intellectual and physical growth of children (Oberhelman *et al*, 1998).

Infection with intestinal helminthes, such as *Ascaris* and hookworm, induces expansion of the Th2 lymphocyte subset, which results in IgE antibody production, eosinophillia and mastocytosis (Pritchard *et al*, 1995; Cooper *et al*, 2000). Th2 responses has been associated with protection against helminth infections, and may counterbalance potentially damaging Th1 responses (Pritchard and Brown, 2001). In contrast, helminth infections may impair immune responses to viruses, bacteria or protozoa, even

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oral vaccines through an antagonistic effect of Th2 cytokines on the expansion of the Th1 lymphocyte subset (Cooper *et al*, 2000).

Immunopathology of intestinal parasites has been studied extensively in experimental animals. However, it has not been fully elucidated, whether Th2 dominant imminunopathological responses, which are found in experimental animals, predominate in people living in helminth endemic areas. In the present study, we analyzed expressions of cytokines (IFN-γ, IL-5 and IL-13) and monosaccharide transporters (GLUT-1 and 5) in the duodenal mucosa of patients from Nong Khai, a northeastern province of Thailand. GLUT-1 is a transporter expressed almost in every tissue. Its expression has been reported to have a strong association with cellular glycolysis, which is accelerated together with up-regulation of GLUT-1 after various cellular stresses, including hypoxia (Dominguez *et al*, 1996; Spolarics, 1996). GLUT-5, on the other hand, is localized to the brush border of intestinal absorptive cells, and has an important role in the absorption of fructose from the intestinal lumen (Dyer *et al*, 2002).

MATERIALS AND METHODS

Biopsy specimens

Biopy specimens were taken from the antrum of the stomach and duodenum of a total of 33 patients who visited Nong Khai Hospital for gastrointestinal problems, such as abdominal discomfort, abdominal pain, vomiting and melena, of various levels of severity and duration. According to Nong Khai Hospital regulations, written consent was obtained from all subjects needing of fiberoptic gastroscopy for diagnostic measures and for duodenal mucosal biopsy for the purpose of studying glucose transporters and cytokine expression. We did not perform any duodenal biopsies on nonsymptomatic or symptomatic patients for whom the fiberoptic examination was unnecessary. Stool examination for parasite ova was performed on the day or several days before the fiberoptic examination by the thick and thin smear method.

Tissue processing

Duodenal mucosa 3 cms distal to the papilla Vater was biopsied, one piece per subject.

Table 1
Five leading diseases in the IPD of Nong Khai Hospital in 1999, 2000 and 2001.

1999	%	2000	%	2001	%
Diarrhea	4.6	Diarrhea	3.5	Diarrhea	2.6
DM	2.4	HT	1.3	DM	1.7
Pneumonia	1.9	DM	1.3	Pneumonia	1.0
HT	1.4	Pneumonia	1.3	CRF	1.0
Head Injury	1.3	Head Injury	1.1	HT	0.9

Table 2
Stool examination in Nong Khai Province.

Year	N	*Opisthorchis viverrini* (%)	Hookworm (%)	Other parasites (%)
1999	8,496	4.9	11.7	23.6
2000	4,110	4.3	7.2	12.9
2001	9,406	9.0	5.1	19.2

Stomach tissue was biopsied to perform the urea-test. The specimens were immediately immersed in 1 ml of RNA preservation solution (RNA later) and kept in a refrigerator for 4-50 days (average 22.6 days) until used for RNA extraction. Total RNA was extracted using TRIZOL reagent (Life Technologies, Rockville, MD). Five-μg aliquots of RNA were reverse transcripted in 20 μl of reverse transcription buffer containing 5 mM $MgCl_2$, 1 mM dNTP mixture, 1 U/ml RNase inhibitor, 0.25 U/ml AMV reverse transcriptase and 0.125 μM oligo dT primer (Takara RNA LA PCR kit, Takara Biomedicals, Osaka, Japan) at 42ºC for 50 minutes.

Polymerase chain reaction (PCR)

One-μl aliquots of synthesized cDNA were added to PCR buffer containing 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.025 U/ml LA Taq DNA polymerase (Takara RNA LA PCR kit), and 0.2 mM of sense and antisense primers, for a final volume of 25 ml. PCR was carried out with cycles of 1 minute at 94ºC, 1 minute at 62ºC and 1 minute at 72ºC. The sense and antisense primers used are shown in Table 5. To determine the optimal numbers of PCR cycles, densities of electrophoresed PCR product from one representative sample were analyzed at different PCR cycles, and fixed numbers of PCR cycles, to allow a comparison between the levels of gene expression determined.

Density analyses of PCR products

Eight μl of the amplified product was electroforesed on agar and stained with ethidium bromide. The fluorescence images were saved by a CCD camera-image saver (ATTO incorporation, Tokyo, Japan), and the density of each band was analyzed by NIH Image software. Band densities were normalized relative to those of bactin bands.

RESULTS

A total of 33 patients were evaluated for glucose-transporter and T cell-cytokine expression in the duodenal mucosa. All the patients were residents of Nong Khai Province, where agriculture is the major industry, and visited the Nong Khai General Hospital from December 2001 to February 2002 because of gastrointestinal symptoms, such as abdominal discomfort, abdominal pain, vomiting or melena. Esophagogastroduodenal fiberoptic examinations were carried out to identify peptic ulcers (6), esophagitis (4), erosive gastritis (3), chronic gastritis (17) and no particular findings (3). Stool examination for parasites or ova performed on the day or several days before fiberoptic examination revealed *Strongyloides* larvae in 2 patients and *Taenia* ova or plogrottids in 2 patients. Past history revealed 2 patients with *Ascaris* infection and 4 patients with *Taenia* infection within the past 6 months. The urea test for *Helicobacter pylori* infection at the time of the study or in the past 2 months. The data for 16 subjects, including age, sex and laboratory data for albumin, he-

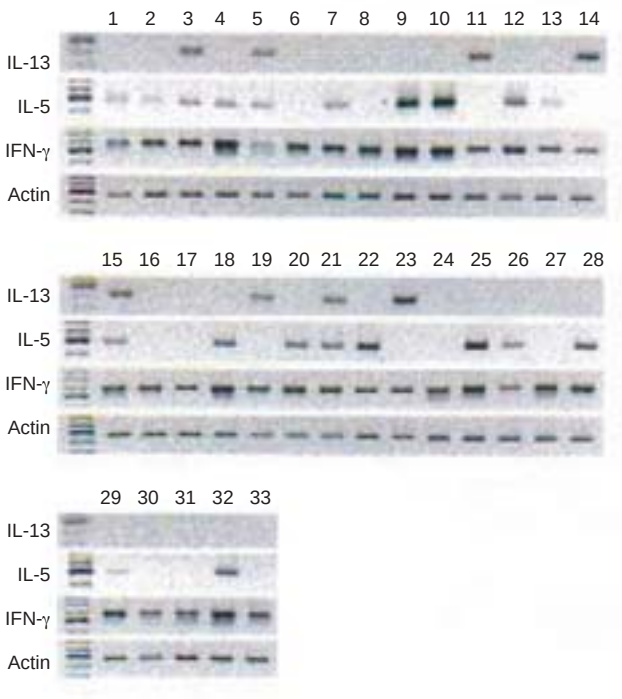

Fig 1–Expressions of IL-13, IL-5 , IFN-γ and β-Actin in the duodenal mucosa of 33 patients. Numbers 1-10: patients with present or recent helminth infection. Numbers 11-26: patients with neither helminth infection nor urea test positivity. Numbers 27-33: urea test-positive patients.

Table 3
Primers and optimal cycles for PCR amplification.

	Primers	Expected product (bp)	Optimal PCR cycle
β-Actin	S 5′-TCAGAAGGATTCCTATGTGGGC-3′	317	22
	A 5′-CCATCACGATGCCAGTGGTA-3′		
GLUT-1	S 5′-ATCGTCAACACGGCCTTCAC-3′	458	32
	A 5′-AAGCCGGAAGCGATCTCATC-3′		
GLUT-5	S 5′-GGTACAACGTGGCTGCTGTC-3′	347	28
	A 5′-CATGGGGACCACGTTGGAAG-3′		
IFN-γ	S 5′-GGGTTCTCTTGGCTGTTACTG-3′	384	34
	A 5′-GACAGTTCAGCCATCACTTGGA-3′		
IL-5	S 5′-GAAATTCCCACAAGTGCATTGG-3′	335	38
	A 5′-CTTTCTATTATCCACTCGGTGTTC-3′		
IL-13	S 5′-AGGAGCTGGTCAACATCACC-3′	296	40
	A 5′-GTTGAACCGTCCCTCGCGAA-3′		

Table 4
Laboratory data of the subjects.

Laboratory data	Total n=33	Helminth[a] 10	*H. pylori*[b] 7	None[c] 16	Ulcer[d] 6	Non-ulcer[e] 27
Age	45.0±13.5	41.9±10.9	43.6±14.4	47.4±14.7	47.0±10.2	44.6±14.2
Sex (M/F)	24/9	10/0	5/2	9/7	5/1	19/8
Albumin (g/dl)	3.9±0.7(26)[f]	4.1±0.6(9)	4.2±0.4(5)	3.7±0.8(11)	3.5±0.8(6)	4.1±0.6(20)
Hemoglobin (g/dl)	13.1±2.7(31)[f]	13.5±2.0(9)	15.1±2.0(7)	12.0±2.8(15)	12.3±4.1(6)	13.3±2.3(25)
Eosinophil (%)	4.9±4.7(30)[f]	5.3±4.7(9)	2.4±2.1(6)	5.6±5.3(15)	2.1±1.9(6)	5.6±5.0(24)

The patients were divided into 3 groups according to infection status: a, Intestinal helminth-positive group which included 2 strongyloidiasis, 2 taeniasis and 6 patients with infection with ascariasis or taeniasis within the last 2 months; b, patients with *H. pylori* infection with a positive urea test, c, no infection group, who did not have evidence of either helminth or *H. pylori* infection. The patients were divided into 2 groups: those with a peptic ulcer (d) and without a peptic ulcer (e). [f]Data on serum albumin, hemoglobin and peripheral blood eosinophil% were not obtained from all 33 patients: the numbers examined are shown in parenthesis. Data shown are means ± S.D.

Table 5
IL-5 and IFN-γ expression levels in the duodenal mucosa.

n	Helminth[a] 10	*H. pylori*[b] 7	None[c] 16	Ulcer[d] 6	Non-ulcer[e] 27
IL-5[f]	0.38±0.19	0.09±0.08[i]	0.27±0.09	0.14±0.08	0.29±0.08
IFN-γ[f]	0.77±0.18	0.84±0.27	0.89±0.17	0.64±0.30	0.89±0.12
IL-5/IFN-γ[g]	0.50±0.18	0.06±0.04[h]	0.30±0.11	0.28±0.17	0.32±0.09

[a-e]See legend for Table 4; data shown are means ± SE. [f]Each mRNA expression level was standardized to that of β-Actin; [g]IL-5 / IFN-γ ratio was calculated using β-Actin-standardized values for IL-5 and IFN-γ; [h]Significantly different from helminth infection group (p=0.047). [i]p =0.211 *vs* helminth group.

moglobin, and peripheral blood eosinophils (%) is shown in Table 4. Hemoglobin concentration level was significantly higher in the urea test-positive group than in the non-infected group, while other laboratory data showed no difference among the groups.

To determine mucosal immunopathological status in gastrointestinally symptomatic patients, mRNA expressions of IFN-γ, IL-5 and IL-13 were examined by RT-PCR. Th2 cytokines IL-5 and IL-13 were not detectable in all the patients, while the Th1 cytokine IFN-γ was detectable in all the patients (Fig 1). The numbers of IL-5-positive and IL-13-positive patients were 20/33 (60.6%) and 8/33 (24.2%), respectively. Prolongation of the number of PCR cycles up to 45 did not increase the number of positive patients. Positive or negative results for IL-5 or IL-13 expressions in individual patients showed no correlation with age, sex, or the period of sample preservation (4-50 days, average 22.6 days) (data not shown). The prevalence of IL-5 and IL-13 expression also showed no significant correlation with infection status (Fig 6). On the other hand, IL-5 expression revealed a significantly higher prevalence in patients with high peripheral blood eosinophil counts (>4.0%) than in low peripheral blood eosiniphil counts (Fig 2a). For semi-qualtitative analyses of PCR results, IL-5 and IFN-γ expressions were standardized to that of β-Actin, and each expression level was determined. IL-5 and IFN-γ expression levels showed no significant correlation with infection status, suggesting that the mucosal Th1/Th2 balance is different between those with helminth infection and those with *H. pylori* infection (Table 5). The presence

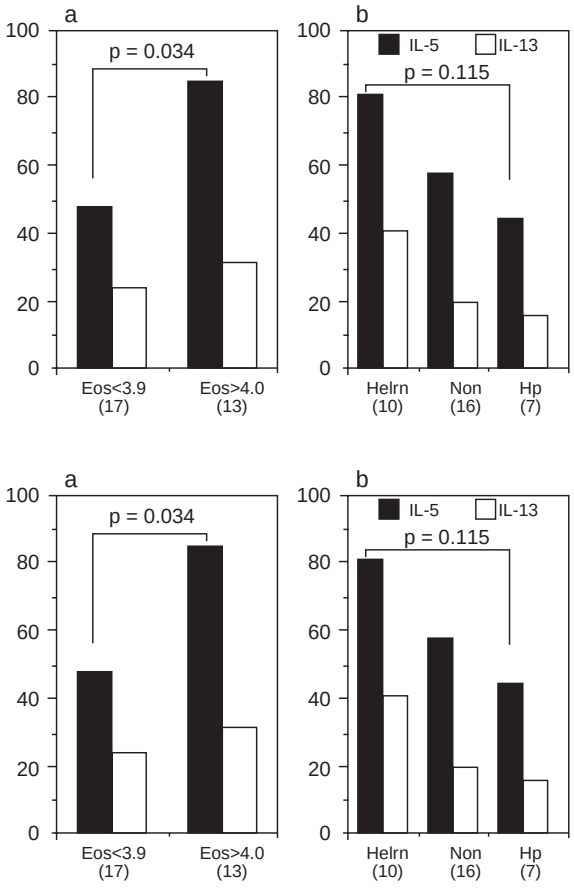

Fig 2–Expression of IL-5 (closed column) and IL-13 (shaded column) in the duodenal mucosa. (a) IL-5 and IL-13 positive rates in patients with peripheral blood eosinophil numbers <3.9 and >4.0%. (b) IL-5 and IL-13 positive rates in patients with present or recent helminth infection (Helm), urea test-positive patients (Hp) or non-positive patients (Non).

Positive rate = 100 x number of subjects expressing IL-5 or IL-13 / total number of subjects. Numbers in parenthesis.

Table 6
GLUT-1 and GLUT-5 glucose transporter expressions in the duodenal mucosa.

	Helminth[a]	*H. pylori*[b]	None[c]	Ulcer[d]	Non-ulcer[e]
n	10	7	16	6	27
GLUT-1[f]	1.00±0.17	0.81±0.14	1.21±0.22	0.89±0.21	1.10±0.14
GLUT-5[f]	1.15±0.14	0.95±0.13[g]	1.30±0.10	1.18±0.16	1.18±0.08

[a-e]See legend for Table 4; data shown are means ± SE; [f]mRNA expression level of GLUT-1 and GLUT-5 were standardized to that of β-Actin. [g]Significantly different from corresponding non-infection group ($p<0.05$).

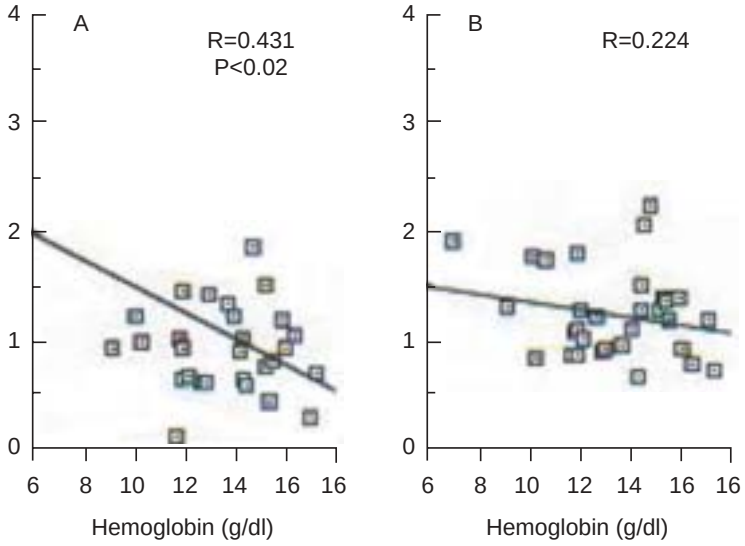

Fig 3–Correlation of peripheral blood hemoglobin concentration and mRNA expression levels of GLUT-1(A) and GLUT-5 (B) in the duodenal mucosa. Expression levels were normalized to that of β-Actin.

or absence of peptic ulcers showed no significant effects on the expression of IL-5 and IFN-γ (Table 5).

Next, we analyzed the expression of glucose transporters GLUT-1 and GLUT-5 in the duodenal mucosa. GLUT-1 and GLUT-5 were detectable in all the patients, and their expression levels were not affected by age, sex, or the period of sample preservation (data not shown). Semi-qualtitative analyses showed that mRNA expression levels of the brush border transporter GLUT-5 were significantly lower in the urease test positive group than in the non-infection group, whereas its expression in the helminth-infection group showed no significant difference from those in the non-infection group (Table 6), suggesting that *H. pylori* infection caused more severe injury to absorptive cells than helminth infections did. Expressions of a ubiquitous glucose transporter GLUT-1 showed no significant association with helminth infection or urease test positivity, although its expression showed an inverse relationship with peripheral blood hemoglobin (Fig 3). Serum albumin concentration or peripheral blood eosinophilia showed no significant correlation either with GLUT-1 or GLUT-5 expression (data not shown). The presence of peptic ulcers showed no significant effect on glucose transporter expression in the duodenal mucosa (Table 6).

DISCUSSION

Mucosal inflamamation appears to be modulated, at least partly if not exclusively, by cytokine responses in the mucosa. Up-regulations of certain cytokine expressions in the duodenal mucosa have been reported in some diseases: IL-5 in food allergy patients (Vandezande *et al*, 1999), IL-3, IL-5 and GM-CSF in asthmatics (Wallaert *et al*, 1995), IFN-γ, IL-2, IL-4, IL-6 and tumor necrosis factor in celiac disease (Nilsen *et al*, 1998), and increases in the numbers of IFN-γ and IL-4 secreting cells in cow's milk sensitive enteropathy (Hauer *et al*, 1997), while IL-5 was undetectable in the duodenal mucosa of healthy individuals (Vandezande *et al*, 1999; Wallaert *et al*, 1995). Infections with intestinal helminthes, such as *Ascaris* and *Strongyloides,* have been reported to induce type 2 cytokine production in peripheral blood mononuclear cells (Cooper *et al*, 2000; Porto *et al*, 2001), but cytokine expression in local tissues, such as the duodenal mucosa, has not been elucidated. In the present study, mRNA positivities for Th2 cytokines IL-13, IL-5 and Th1 cytokine IFN-γ in the duodenal mucosa were 24.2, 60.6, and 100%, respectively. Positivities of IL-5, an important factor of eosinophil differentiation, proliferation, survival and migration, showed a significant association with levels of peripheral blood eosinophilia. In addition, helminth-infection groups showed the highest positivities of IL-5 in the duodenal mucosa (80.0%). In contrast, the urea test-positive group showed significantly suppressed levels of IL-5

expression as well as the ratio of IL-5 to IFN-γ expression compared to those in the helminth-infection group. The results are consistent with previous reports that *H. pylori* infection induced type 1 cytokines in gastric mucosal T cells with little production of type 2 cytokines (D' Elios *et al*, 1997; Bamford *et al*, 1998). This suggests that mucosal immunological status is regulated differently between helminth and *H. pylori* infection even in patients in helminth endemic areas.

The present results show that substantial numbers in the non-infection group also showed positive IL-5 expression (56.2%). The non-infection group consisted of individuals who had certain gastrointestinal symptoms, but had neither a recent history of helminth infection nor urease test positivity. However, since those subjects were from the rural area where overall parasite ova-positive rates are 5.1-11.7% (Table 2), it is likely that those subjects were highly heterogeneous in terms of a past history of intestinal infections, including many subjects who had been infected on and off with some species of helminth.

Transporters of monosaccharides, amino acids and peptides have important roles in nutritional absorption from the intestine and alteration in the expression may exert a deleterious effect on the host. In the present study we found that expression of GLUT-5, a brush-border monosaccharide transporter, was slightly, but significantly decreased in the urease test-positive group. *H. pylori* bacilli primarily colonize the gastric epithelial cell surface, and the duodenum, especially in areas of gastric metaplasia in the duodenum (Kozol and Dekhne, 1994). There are several mechanisms of down-regulation of GLUT-5 expression in subjects with a positive urease test. First, it is possible that a part of the biopsied duodenal mucosa may revealed gastric metaplasia, which does not express GLUT-5; second, *H. pylori* bacilli, if they colonize the duodenal mucosa, may cause intense cytopathic effects on absorptive cells, and third; *H. pylori* infection in the gastric mucosa might have induced extensive immunopathological effects not only in the stomach, but also in the duodenum. In contrast, we found no significant alteration of GLUT-5 mRNA expression in the helminth-infection group, suggesting that *H. pylori* infection may be a more common deleterious factor on the function of absorptive cells than helminth infection.

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