Gastric inflammatory markers and interleukins in patients with functional dyspepsia treated with astaxanthin

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Abstract

The chronic active inflammation caused by *Helicobacter pylori* is dominated by neutrophils, macrophages, lymphocytes and plasma cells. Several interleukins are involved in the inflammatory process. The aim of this study was to investigate the effect of astaxanthin on gastric inflammation in patients with functional dyspepsia. Forty-four consecutive patients were included, and biopsies were examined for IL-4, IL-6, IL-8, IL-10, interferon-γ, CD4, CD8, CD14, CD19, CD25 and CD30. Patients were randomized: 21 patients were treated with 40 mg of astaxanthin daily, and 23 patients were treated with a placebo. There was a significant decrease in gastric inflammation in *H. pylori*-positive patients from both groups. There were no significant changes in the density of *H. pylori* or in any of the interleukins during or after treatment. There was a significant up-regulation of CD4 and down-regulation of CD8 in patients with *H. pylori* treated with astaxanthin. Astaxanthin had an effect on the inflammation and on the density of *H. pylori* in mice in a study where the diet could be standardized without antioxidants (Bennedsen *et al.*, 1999). These dietary conditions are impossible in studies involving humans, and may be due to the minor effect when the host have access to antioxidants in their diet.

Introduction

Helicobacter pylori is the most important cause of chronic active gastritis and gastric and duodenal ulcers, and plays an important role in the development of gastric cancer and mucosa associated lymphoid tissue (MALT) lymphomas (Graham *et al.*, 1991; Parsonnet *et al.*, 1991; Kreiss *et al.*, 1995). The acute *H. pylori* infection induces mucosal infiltration dominated by neutrophil leucocytes initiated by invasive *H. pylori* or water-soluble proteins passing the mucosal barrier (Bode *et al.*, 1987; Andersen & Holck, 1990; Enders *et al.*, 1995). Within a few weeks, the acute inflammation develops to chronic active inflammation dominated by neutrophils, macrophages (CD14), lymphocytes (CD4, CD8 and CD19) and plasma cells (Bode *et al.*, 1987; Andersen & Holck, 1990; Dixon *et al.*, 1996). Several interleukins are involved in the inflammatory process in the gastric mucosa. Production of interleukin-8 (IL-8), a neutrophil chemotactic factor, by gastric epithelial cells is stimulated by *H. pylori* (Noach *et al.*, 1994; Fan *et al.*, 1995; Ando *et al.*, 1996). An enhancement of interferon-γ (IFN-γ) production by T-cells stimulated by *H. pylori* is often found (Bamford *et al.*, 1998; Ren *et al.*, 2000). The anti-inflammatory cytokine IL-10 inhibits the synthesis of proinflammatory cytokines and is stimulated by *H. pylori in vitro* and *in vivo* (de Waal Malefyt *et al.*, 1991; Mohammadi *et al.*, 1996; Bodger *et al.*, 1997; Hida *et al.*, 1999). The majority of studies on cytokine responses to *H. pylori* have been investigated in cell cultures and animal models.

It has previously been shown that CD4, CD14 and CD19, IFN-γ, IL-8 and IL-10 increase significantly with increasing inflammation and increasing *H. pylori* density in patients with peptic ulcers or severe gastritis (Holck *et al.*, 2003), whereas only CD4 and CD19 were significant correlated with inflammation and *H. pylori* density in patients with mild inflammation (Andersen *et al.*, 2005). The antioxidant carotenoid astaxanthin (Higuera-Ciapara *et al.*, 2006) produced by the micro-alga *Haematococcus pluvialis* has been shown to reduce both the inflammation and the density of

H. pylori in the stomach of *H. pylori*-infected mice (Bennedsen *et al.*, 1999).

The aim of this study was to investigate gastric inflammation, inflammatory markers (interleukins and CD cell markers) and density of *H. pylori* in patients with functional dyspepsia and mild gastritis following treatment with astaxanthin.

Materials and methods

Patients

Forty-four consecutive patients (six males, 38 females; median age 51 years, range 20–70 years) with functional dyspepsia admitted to endoscopy at Kaunas University of Medicine, Lithuania were included in the study. All patients gave informed consent and the study was in accordance with the second Helsinki declaration and approved by the local ethics committee. None of the patients had received antibiotics, bismuth compounds, H_2 antagonists, proton-pump inhibitors or immune-modulating drugs within the 4 weeks before endoscopy.

Treatment

The patients were randomized into two groups and treated blindly with (1) five placebo capsules twice daily (23 patients: three males, one female, median age 45 years, range 20–70 years) or (2) five capsules, each with 4 mg astaxanthin, twice daily (21 patients: three males, 18 females, median age 52 years, range 29–66 years). The placebo capsules were filled with 400 mg dextrin, and the active capsules were filled with 290 mg dextrin and 110 mg astaxanthin rich alga meal from *Haematococcus pluvialis* (Astacarox, BioReal AB, Sweden), corresponding to 4 mg pure astaxanthin.

Biopsies

During endoscopy three gastric antral biopsies were obtained: one was formalin-fixed for histological examination, and two were frozen in liquid nitrogen for culture and immunohistochemistry.

Morphological examination

Formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin and examined for morphological changes according to the revised Sydney classification of gastritis (Dixon *et al.*, 1996). The type of gastritis was recorded, and the degree of gastritis was scored semi-quantitatively from 0 (absent) to 3 (extensive) blinded and independently by two experienced pathologists.

Detection of *Helicobacter pylori*

(1) All patients took a urea breath test (UBT) before the endoscopy. (2) The density of *Helicobacter*-like organisms (HLO) in histological sections stained with antibodies to *H. pylori* was scored semi-quantitatively from 0 (absent) to 3 (extensive). (3) Biopsies frozen in liquid nitrogen were cultured under microaerophilic conditions (5% O_2) on chocolate agar plates containing 7% lysed and defibrinated horse blood at 36 °C for up to 10 days.

Cell markers and cytokines

Sections of biopsies frozen in liquid nitrogen were stained with monoclonal antibodies recognizing the cytokines (R&D Systems) and cell markers (DakoCytomation, Denmark) as primary antibodies, and immunostaining was performed using a sensitive biotin-avidin immunoperoxidase method (ABComplex/HRP staining procedure, DAKO, Denmark). The sections were counterstained with hematoxylin to enhance morphological details. The sections were examined for IL-4, IL-6, IL-8, IL-10 and IFN-γ as well as for CD4, CD8, CD14, CD19, CD25 and CD30. The density of cytokines and of cell markers were scored semi-quantitatively on a scale from 0 (absent) to 3 (severe), blinded, by one experienced pathologist, twice separately. The results for each group of patients were described as an average score index, which is the average score times 100.

Statistical analysis

Fisher's exact test was used to verify a significant association between inflammatory activity, *H. pylori* density, cytokines and cell markers. $P < 0.05$ was regarded as significant.

Results

Helicobacter pylori status and morphology

Twenty-nine patients were positive for *H. pylori* and 15 patients were negative for *H. pylori* by UBT, histology or culture. Fifteen patients with *H. pylori* and eight patients without *H. pylori* were included in the group treated with 40 mg astaxanthin daily, and fourteen patients with *H. pylori* and seven without *H. pylori* were included in the placebo group. All fifteen *H. pylori*-negative patients had no or only mild chronic inflammation. The *H. pylori*-positive patients had mild, moderate and severe chronic inflammation in addition to no, mild or moderate active gastritis. A significant correlation was found between the presence of *H. pylori* and the presence of inflammation, as well as between the density of *H. pylori* and the degree of inflammation, as previously described (Andersen *et al.*, 2005). CD4 and CD19 were the only cell markers significantly correlated with gastric inflammation and the presence of *H. pylori*, as previously described

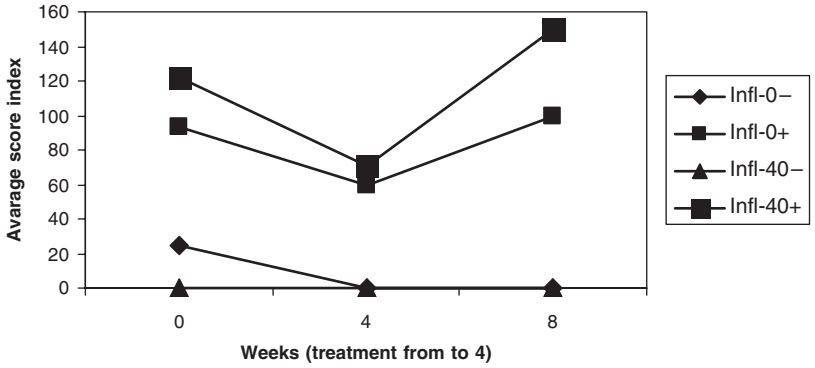

Fig. 1. Degree of inflammation in gastric mucosa from patients with and without *Helicobacter pylori* and treated with 40 mg astaxanthin daily or a placebo. Average index score = average score × 100. Inf-0 – : inflammation in *H. pylori*-negative patients treated with the placebo. Inf-0+: inflammation in *H. pylori*-positive patients treated with the placebo. Inf-40 – : inflammation in *H. pylori*-negative patients treated with astaxanthin. Inf-40+: inflammation in *H. pylori*-positive patients treated with astaxanthin.

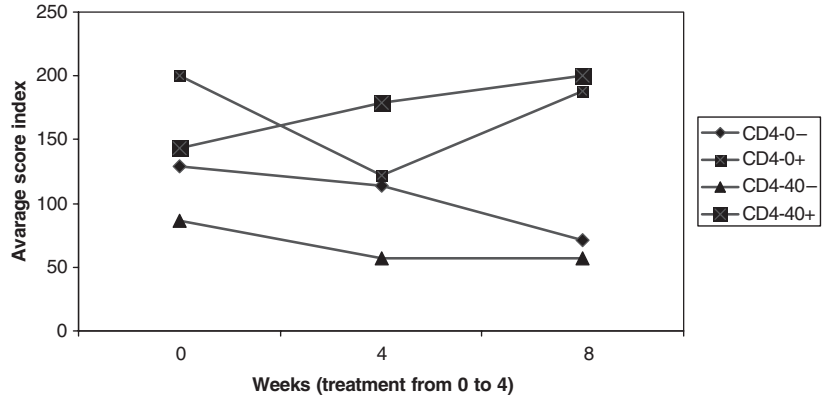

Fig. 2. Level of CD4 in gastric mucosa from patients with and without *Helicobacter pylori* and treated with 40 mg astaxanthin daily or a placebo. Average index score = average score × 100. CD4–0 – : CD4 in *H. pylori*-negative patients treated with the placebo. CD4–0+: CD4 in *H. pylori*-positive patients treated with the placebo. CD4–40 – : CD4 in *H. pylori*-patients treated with astaxanthin. CD4–40+: CD4 in *H. pylori*-positive patients treated with astaxanthin.

(Andersen *et al*., 2005). In addition, nonsignificant increases were found for IL-8, IL-10 and IFN-γ.

Effect of treatment with astaxanthin

There was a decrease in the inflammation of *H. pylori*-positive patients during treatment. The decrease was significant both in patients treated with astaxanthin and in those treated with the placebo (Fig. 1). After treatment there was a significant increase in the inflammation of *H. pylori*-positive patients. There were no significant changes in the density of *H. pylori* during or after treatment. No significant changes were observed in any of the interleukins during or after treatment. CD4 was significantly upregulated (< 0.05) in patients with *H. pylori* when treated with 40 mg of astaxanthin daily, and significantly downregulated (< 0.001) in patients with *H. pylori* treated with the placebo (Fig. 2). There were no significant changes in *H. pylori*-negative patients. CD8 was significantly downregulated ($P < 0.01$) in patients with *H. pylori* when treated with 40 mg of astaxanthin daily, whereas no changes were observed in patients with *H. pylori* treated with the placebo (Fig. 3). A significant decrease ($P < 0.01$) in CD8 was observed during treatment with the placebo in patients without *H. pylori*. There were nonsignificant up- or down-regulations in several other interleukins or CD markers (data not shown), but not in a way that could be described with an inflammatory index.

Discussion

Most previous studies on cytokines and cell markers of mucosal inflammation have focused on patients with severe gastritis or peptic ulcers (Bamford *et al*., 1998; Hida *et al*., 1999; Ren *et al*., 2000; Holck *et al*., 2003). In this study a group of patients with functional dyspepsia without major gastric diseases such as peptic ulcers or gastric cancer were

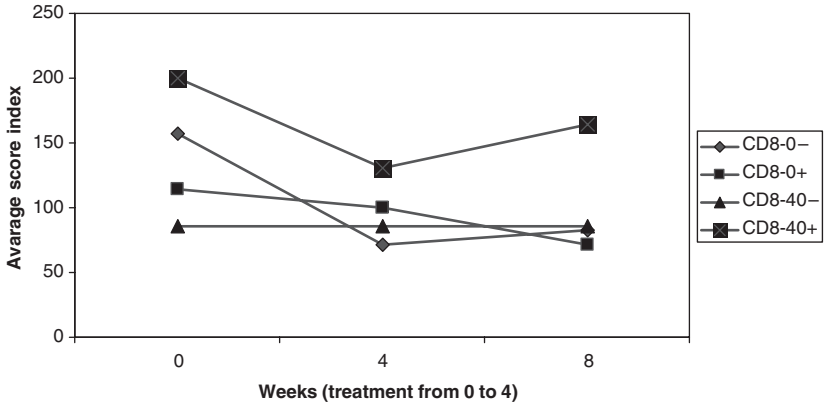

Fig. 3. Level of CD8 in gastric mucosa from patients with and without *Helicobacter pylori* and treated with 40 mg astaxanthin daily or a placebo. Average index score = average score × 100. CD8–0 – : CD8 in *H. pylori*-negative patients treated with the placebo. CD8–0+: CD8 in *H. pylori*-positive patients treated with the placebo. CD8–40 – : CD8 in *H. pylori*-negative patients treated with astaxanthin. CD8–40+: CD8 in *H. pylori*-positive patients treated with astaxanthin.

examined before, during and after treatment with the antioxidant astaxanthin, which had not been done before in dyspeptic patients treated with astaxanthin. The purpose was to describe changes in the inflammation of the gastric mucosa in patients with and without *H. pylori* infections when treated with astaxanthin or a placebo.

In accordance with many previous studies there was a close association between the degree of inflammation and the density of *H. pylori* (Noach *et al.*, 1994; Kreiss *et al.*, 1995; Dixon *et al.*, 1996; Holck *et al.*, 2003; Andersen *et al.*, 2005). A down-regulation of the inflammation was observed in the group of *H. pylori*-positive patients during treatment either with astaxanthin or with a placebo. After treatment, a reactive up-regulation of the inflammation was observed in both groups (Fig. 1). Thus, the effect on the inflammation was not caused by astaxanthin but could be explained by some compounds of the capsules or the dextrin, had an anti-inflammatory effect. Alternatively it might be caused by a psychological effect arising from the expectation of being treated. Treatment of patients with astaxanthin had no effect on the density of *H. pylori*.

The T-helper cell marker, CD4 (Th), and the B cell marker, CD19, were significantly correlated with the degree of inflammation and the density of *H. pylori*, indicating that the response to *H. pylori*, even in patients with functional dyspepsia and with mild inflammation, is predominantly a humoral immune response and not a cytotoxic response, as CD8 (Tc) markers were independent of the inflammation and of the density of *H. pylori*. However, during treatment a significant up-regulation of CD4 was observed in patients treated with astaxanthin, and a significant down-regulation was observed in patients treated with the placebo. In addition, CD8 was downregulated significantly during treatment with astaxanthin but not with the placebo. These changes indicate a further shift towards a humoral immune response rather than a cytotoxic response. This was not, however, reflected in the immunoglobulin G (IgG) antibody response (data not shown). There were trends in up- or down-regulation of other interleukins and CD markers, but the variations between the four groups of patients were too great and the number of patients in each group was too small to show any significant changes. These trends were not reflected when indices for total interleukins or total CD markers or a total inflammatory index, as previously described (Andersen *et al.*, 2005), were used.

In this study the most marked effect of astaxanthin was seen in the CD4/CD8 response, but the changes were not reflected in the humoral immune response. In animal models, where the diet can be standardized without antioxidants such that the animals do not experience any antioxidant stress, astaxanthin had a tremendous effect on inflammation and on the density of *H. pylori* (Bennedsen *et al.*, 1999; Eaton *et al.*, 2001). This is impossible in human studies, and it seems that treatment with antioxidants is less effective in studies where the hosts have access to antioxidants through diet.

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